Biosorption of Pb(II) by bacterial strain *Bacillus badius* AK isolated from rotary drum compost of water hyacinth

Thesis submitted in partial fulfilment of the requirements for the award of the degree of

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by

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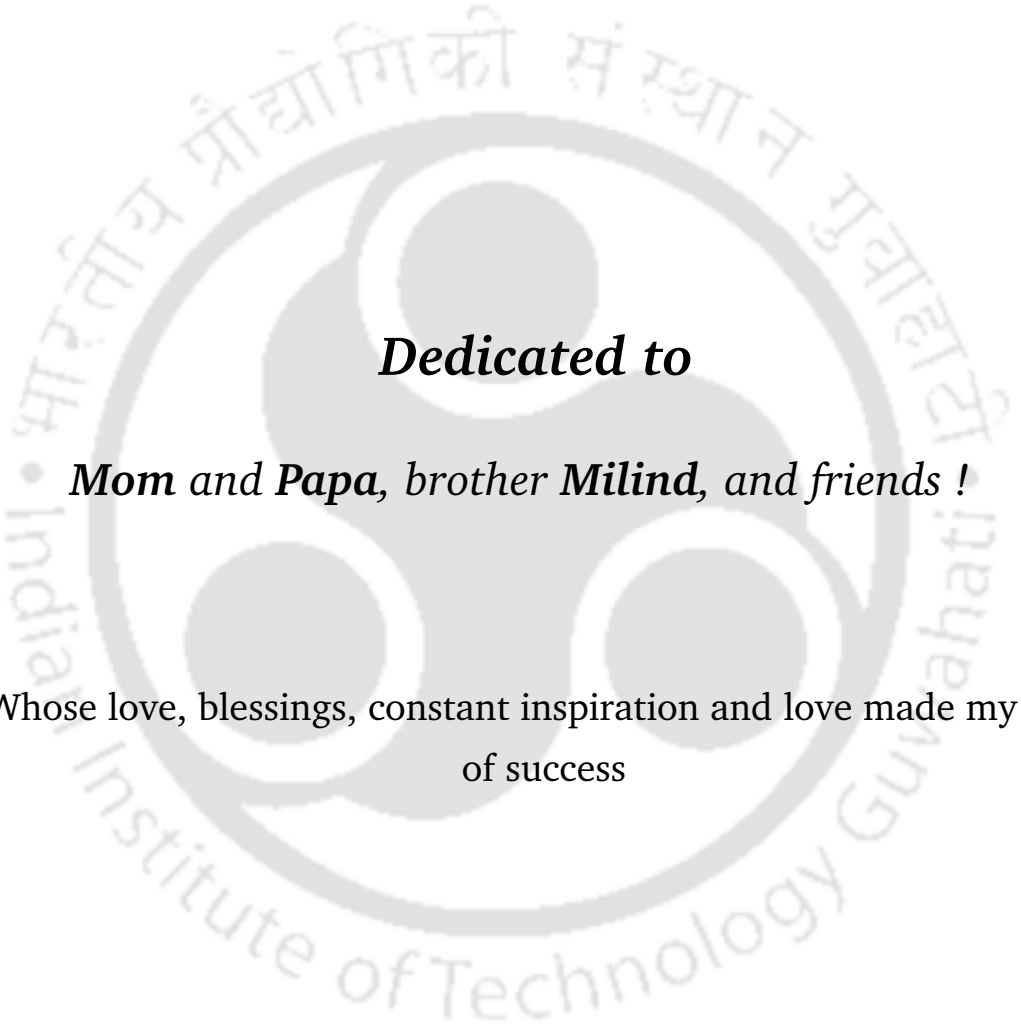

Dedicated to

***Mom** and **Papa**, brother **Milind**, and friends !*

Whose love, blessings, constant inspiration and love made my path of success

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This is to certify that this thesis entitled "**Biosorption of Pb(II) by bacterial strain *Bacillus badius* AK isolated from rotary drum compost of water hyacinth**" submitted by **Isha Vishan** (Roll No. 126152001), in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy, to the Indian Institute of Technology Guwahati, Assam, India, is a record of the bonafide research work carried out by her under my guidance and supervision at the Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India. To the best of my knowledge, no part of the work reported in this thesis has been presented for the award of any degree at any other institution.

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Abstract

Composting of biological waste is mainly governed by the diversity of microorganisms working intermittently or in succession in order to carry out the biochemical reactions for their metabolism. Presence of certain desirable microbial communities is reported to enhance the composting process. The rotary drum composting is relatively a faster method as compared to the conventional pile (windrow) composting method. The water hyacinth (*Eichhornia crassipes*) being a free-floating aquatic weed is creating nuisance in waterbodies, composting is found to be one of the most effective method of management and utilization of this weed. From the previous studies it has been found that the total content of metal has increased to a significant amount in final compost of water hyacinth. Therefore, current study was performed to detect the microbial succession in the rotary drum composting of water hyacinth along with the stability and maturity analysis. Different ratios of water hyacinth, cow dung and sawdust such as, 8:1:1, 7:2:1, 6:3:1, 5:4:1 and 10:0:0 (control), respectively were used for rotary drum composting. Major microbial communities in the best trial (6:3:1) of water hyacinth compost were observed to be 39.08% of *Bacteroidetes*, 24.21% of *Flavobacteria* followed by 24.21% of *Flavobacteriales*. *Flavobacterium* genus was found to be most abundant in the composting process. Several successful studies have been reported regarding the isolation and characterization of microbes from various waste sources such as wastewater, soil, sewage sludge, industrial waste and compost followed by the utilization of microbes in micro-bioremediation with biosorption process. However, no work has been reported so far in relation to isolation and identification of bacteria during rotary drum composting of water hyacinth. Therefore, in this thesis work rotary drum compost of water hyacinth was used as a source for isolation of microbes. The bacterial diversity in the rotary drum composting of water hyacinth was analyzed with culture-dependent and culture-independent techniques. Twelve bacterial isolates were isolated and identified, which majorly belonged to the *Bacillus* and *Entrobacter* genera.

The biosorption study of heavy metals such as lead (Pb(II)) and cadmium (Cd(II)) were performed with isolated bacterial strain *Bacillus badius* AK. Live (non-pretreated) and dried (pretreated) bacterial cells were utilized for biosorption of Pb(II) and Cd(II). Batch biosorption study of live (non-pretreated) biomass of *Bacillus badius* AK demonstrated maximum biosorption of Pb(II) at biomass concentration of 1.7×10^{16} CFU/mL, at 100-150 mg/L concentration of Pb(II) in the solution. The specific growth rate and maximum specific growth rate of bacterial cells under the influence of Pb(II) were determined as 0.05/h and 2.54/h

respectively. The rate of biosorption in batch study with dried (pretreated) biomass of *Bacillus badius* AK was observed to be high in the first 30 min. The maximum adsorption capacity was 138.88 mg (Pb(II)) /g (dried biomass). Pb(II) removal efficiency was observed to be 60% after desorption. Biosorption of Cd(II) by dried biomass of *Bacillus badius* AK was found to have maximum biosorption capacity of 131.58 mg (Cd(II)/g (dried biomass). The recovery of biosorbent after biosorption was 39% of the initial value and the recovery after desorption was 68.2%. The continuous column mode operation of dried biomass of *Bacillus badius* AK in biosorption of Pb(II) was observed with higher breakthrough capacity as compared to the batch process. The maximum biosorption was reported within a span of 30 min, at high flow rate the metal ions get less time for attachment with the binding sites, however, the adsorbent gets exhausted rapidly. At low flow rate, the continuous system is not practically suitable for industrial wastewater treatment plant. Finally, the results indicated that dried form of *Bacillus badius* AK had more biosorption capacity in heavy metal removal than the live (non-pretreated) bacterial form. The dried (pretreated) biomass of *Bacillus badius* AK has much potential as a biosorbent for removal of heavy metals such as Pb(II) and Cd(II) from aqueous solution at lab scale. Additionally, it is an economical and promising substitute of biosorbent for heavy metal removal as compared with the existing conventional methods of biosorption.

Keywords: Rotary drum composting, Water hyacinth, Biosorption, *Bacillus badius* AK

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List of Symbols

Symbols	Description
μ	Specific growth rate
μ_{max}	Maximum pecific growth rate
K_s	Saturation constant
q_e	Metal uptake by biosorbent
q_{max}	Maximum metal uptake at equilibrium
C_i	Initial metal concentration
C_f	Final metal concentration
V	Volume of metal solution
M	Mass of biosorbent
q_e	Adsorption capacities at equilibrium
q_t	Adsorption capacities at time t
k_1	Pseudo-first-order rate constant
k_2	Pseudo-second-order rate constant
χ^2	Chi-square
Σ	Set of events of the RMSE test
K_L	Langmuir constant
C_e	Concentration of metal in solution at equilibrium
K_F	Freundlich constant
$1/n$	Intensity of adsorption
ΔG°	Gibbs free energy
ΔH°	Enthalpy of adsorption
ΔS°	Entropy of adsorption
T	Temperature in Kelvin
R	Gas constant
k_d	Rate constant for intraparticle diffusion parameter
C	Thickness of the boundary layer
W_c	Weight of dried bacterial biomass
V_c	Cylinder volume

Q	Volumetric flow rate
V_t	Throughput volume
t	Function of time
Vn	throughput volume at nth reading
V$_{n+1}$	Throughput volume at (n+1)th reading
C$_n$	effluent adsorbate concentration at nth reading
V$_E$	Throughput volume at exhaustion
C$_0$	Influent adsorbate concentration
I	Influent adsorbate load
M_r	Mass of the adsorbent removed
M	Mass of adsorbate not removed
t_s	Service time at breakthrough point
N_o	Dynamic bed capacity
Z	Packed-bed column depth
u	Linear flow rate
k_{ads}	Adsorption rate constant
Z_o	Critical bed depth

List of Abbreviations

Terms	Abbreviations
L	Litre
mg	Milligram
g	Gram
Kg	Kilogram
M	Molar
OUR	Oxygen Uptake Rate
MC	Moisture Content
CFU/g	Colony Forming Unit/gram
MPN/g	Most Probable Number/gram
VS	Volatile Solids
EC	Electrical Conductivity
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
TC	Total Coliform
FC	Fecal Coliform
DTPA	Diethylene triamine penta-acetic acid
TCLP	Total Characteristic Leaching Procedure
AAS	Atomic Absorption Spectrometer
DNA	Deoxyribonucleic Acid
gDNA	Genomic Deoxyribonucleic Acid
dsDNA	Double Stranded Deoxyribonucleic Acid
RNA	Ribonucleic Acid
QC	Qubit Concentration
PCR	Polymerase chain reaction
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
RPM	Rotations Per Minute
Kcal	Kilo Calories

ND	Not Detected
USEPA	United States Environmental Protection Agency
BIS	Bureau of Indian Standards
WHO	World Health Organization
SEM	Scanning Electron Microscope
EDX	Energy-dispersive X-ray Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
BET	Brunauer-Emmett-Teller
XRD	X-ray powder Diffraction
BDST	Bed Depth Service Time

"Sooner or later, we will have to recognise that the Earth has rights, too, to live without pollution. What mankind must know is that human beings cannot live without Mother Earth, but the planet can live without humans."

Evo Morales

1

Introduction

This chapter comprises a brief discussion about the problems of water hyacinth *(Eichhornia crassipes)* and heavy metals in the environment. The usage of different kind of wastes as a source of microbial isolation, followed by the exploitation of microbes in the removal of heavy metals are also discussed. The chapter also deals with the major objectives, the need of the study, the scope of the thesis and finally thesis organization.

1.1 Overview

The water hyacinth *(Eichhornia crassipes)* is a free-floating perennial weed, a native of South America. It covers around 23.2% wetland area of the northeast region of India, it was introduced in West Bengal as an ornamental plant, but now it has become a nuisance (Abbasi, 1998; Husain, 2003; Shanab et al., 2010). The fast-growing nature and robustness of its seeds causes major problems such as reduction of fishes, blockage of shipping routes, and impeding other irrigational activities (Gunnarsson and Petersen, 2007; SushilKumar, 2011). Water hyacinth also degrades water quality by blocking photosynthesis, takes aquatic nutrients, and invades water bodies. These actions threaten the biodiversity, social and economic systems (Zhang et al., 2010; UNEP, 2013). Literatures are documented with respect to utilization of water hyacinth as a substitute for animal fodder (Tag El-Din, 1992), fermentation feed and biogas production (Singhal and Rai, 2003), pulp and paper industry (Goswami and Saikia, 1994), and the composting and vermicomposting of water hyacinth (Gajalakshmi et al., 2001; Gupta et al., 2007; Singh and Kalamdhad, 2013a,b). Composting of water hyacinth is found to be the most effective method of its management. Composting and vermicomposting are the promising technologies for degrading green waste into stabilized material i.e. compost, which has huge agricultural application as a soil conditioner (Singh and Kalamdhad, 2012). However, a huge amount of non-biodegradable and toxic

heavy metals are found in compost, which hinders its application for agricultural purposes. Compost when added to the soil can lead to accumulation of toxic heavy metals into water, plants and finally enters into the animal and human food chain, in this way it accentuates the problem of bioaccumulation (Iwegbue et al., 2007; Singh and Kalamdhad, 2011). Heavy metals are toxic to plants, soil, aquatic life and human health if their concentration is high in compost. Additionally, these heavy metals exhibit toxic effect on soil biota by affecting their key microbial process and decrease in number and activity of soil microbes (Singh and Kalamdhad, 2011). Heavy metals are carcinogenic and toxic causing biomagnification in terrestrial and marine ecosystems, according to the United States Environmental Protection Agency, some heavy metals such as Ni, Cd, Zn, Cr, Se, Th, Be, As, and Pb are the most hazardous heavy metals (Janssen et al., 1993; Moore and Ramamoorthy, 2012). Zn, Cu, Mn, Fe, Ni, Pb, Cd, and Cr have been found to exist in water hyacinth compost in differently available forms to the plants with varied levels of bioavailability and toxicity (Singh and Kalamdhad, 2012, 2013a). Bioavailability was detected in form of water-soluble fraction, diethylenetriamine penta-acetic acid (DTPA) extractable and toxicity characteristic leaching procedure (TCLP) for leachability analysis (Singh and Kalamdhad, 2013a). The literatures provided the evidence of the changes in total metal concentration and its bioavailability during composting of water hyacinth.

The degradation and stabilization of organic matter during composting process is carried out by microbial communities such as bacteria, actinomycetes, and fungi, they are the primary decomposers in composting (Cunha-Queda et al., 2007; Umsakul et al., 2010; Bhatia et al., 2013). The rigorous process of aerobic degradation of organic waste is the outcome of the metabolic activities by microorganisms, therefore compost serves for the abundance of metabolically active microbes (Hassen et al., 2001; Ryckeboer et al., 2003; Anastasi et al., 2005; Bhatia et al., 2013; Pan and Sen, 2013). Additionally, a high count of actinomycetes, streptomycetes, bacteria and fungi was also observed throughout the whole period of rotary drum composting of water hyacinth. Several successful studies have been reported regarding the isolation and characterization of microbes from various waste sources such as wastewater, soil, sewage sludge and industrial wastes. *Pseudomonas aeruginosa, Bacillus sphaericus, Pseudomonas fluorescens, Microbacterium* sp., *Firmicutes, Actinobacteria, Burkholderia* sp., *Bacillus cereus, Bacillus pumilus, Delftia tsuruhatensis, Proteobacteria*, and *Achromobacter* sp. were isolated from the soil (Jansen et al., 1994; Hassen et al., 1998; Pal and Paul, 2004; Bahig et al., 2008; Sheng et al., 2008; Shoeb et al., 2009; Sun et al., 2010; Çolak et al., 2011; Karelová et al., 2011; Bautista-Hernández et al., 2012; Stanbrough et al., 2013). Microbes such as *Variovorax paradoxus, Rhodococcus* sp., *Flavobacterium* sp. and CCNWRS33-2 were isolated from plant roots grown in sewage sludge and wastewater (Belimov et al., 2005; Wei et al., 2009), *Micrococcus* sp. was isolated from industrial

wastewater (Congeevaram et al., 2007), *Bacillus marisflavi* and *Arthrobacter* sp., were isolated from tannery effluent, electroplating and industrial wastes (Mishra and Doble, 2008). *Pseudomonas* sp. was isolated from uranium mine (Choudhary and Sar, 2009). *Proteobacteria* and *Firmicutes* were isolated from sediment and wastewater (Jose et al., 2011).

Microorganisms and heavy metals are naturally present in the compost environment, the aerobic degradation of organic waste is due to the metabolic activities of microorganisms (Ryckeboer et al., 2003; Anastasi et al., 2005). Also, the microorganisms and heavy metals are naturally present in the environment, they are exposed to each other and it is considered that their interaction is since the beginning of life (Silver and Phung, 1996; Martinez et al., 2009). Microbial metabolism and physiology has acclimatized with the surrounding metal concentration (Kosolapov et al., 2004; Hantke, 2005; Bong et al., 2010). The microbes diversify in different ecological habitats, they have a valuable role in environmental microbiology, eventually, they are exploited in varied microbe-based technologies. Different biomasses originating from soil, industrial wastes, and wastewater have been exploited in live and dead conditions for the removal of heavy metal, some examples of them are such as, *Pseudomonas stutzeri, Bacillus thuringienesis, Bacillus subtilis, Bacillus* sp., *Bacillus* sp. L14, *Streptomyces ciscaucasicus, Streptoverticillium cinnamomeum,* and *Bacillus licheniformis* (Hossain and Anantharaman, 2006; Tunali et al., 2006; Zhou et al., 2007; Oh et al., 2009; Guo et al., 2010; Li et al., 2010; Oves et al., 2013).

Other frequently used bacteria for studying the effect and their removal potential of heavy metals such as Cr, Cd, Ni, Pb, and Zn include *Pseudomonas aeruginosa, Graphium putredinis, Fusarium* sp., *Penicillium chrysogenum, Bacillus sphaericus, Streptoverticillium cinnamoneum, Citrobacter* strain and *Delftia tsuruhatensis* (Puranik and Paknikar, 1997; Alvarez et al., 1999; Puranik and Paknikar, 1999; Pal and Paul, 2004; Bautista-Hernández et al., 2012; del Carmen Vargas-García et al., 2012), *Sargassum* sp., *Padina* sp., *Geobacillus thermodenitrificans* and *Geobacillus thermocatenulatus* for Pb, Cu, Cd, Zn, and Ni removal (Sheng et al., 2004; Babák et al., 2013), *Citrobacter freundii* and *Klebsiella pneumoniae* for Pb removal (Al-Garni, 2005) and *Bacillus* sp. for Pb and Cu removal (Tunali et al., 2006). Fungi used for heavy metal removal include *Ganoderma Lucidium, Aspergillus niger, Rhizopus oryzae, Saccharomyces cerevisiae, Rhizopus arrhizus*, and *Penicillium chrysogenum* (Sağ and Kutsal, 1996; Krishna and Philip, 2005; Park et al., 2005).

Biosorption is one of the most significant indigenous properties of living or dead microbes, relevant for the metal removal purpose (Volesky, 2007; Gadd, 2009; Fomina and Gadd, 2014). Studies have shown that microbes which are resistant to heavy metals can be used for the biosorption and bioaccumulation of the heavy metals from the surroundings (Wang and Chen, 2006; Gadd, 2009). As a part of micro-bioremediation, biomass of bacteria, fungi, and actinomycetes have been extensively studied for biosorption. Hence there

is an immense possibility in using them for biosorption, biotransformation, and/or bioaccumulation of the heavy metals (Anastasi et al., 2005; del Carmen Vargas-García et al., 2012).

There are several physiochemical techniques adopted to remove heavy metals from aqueous solutions which include precipitation, electroplating, ion exchange, evaporation, and membrane process. However, these methods have been found uneconomical and inefficient at low metal concentrations, followed by generation of secondary wastes and large amounts of chemical reagents (Kuyucak and Volesky, 1988; Fomina and Gadd, 2014). The emerging trend of biosorption process for the heavy metal removal is proving to be an efficient technique of micro-bioremediation. The usage of biomass including microbes (bacteria, cyanobacteria, fungi and yeasts etc.), algae, natural residues (sawdust, barks, crab shell etc.), industrial wastes (food wastes, fermentation residue, activated sludge etc.), agricultural wastes (wheat bran, rice husk, fruits and vegetable waste etc.) is practiced in the biosorption process. Biosorbent materials, such as the green algae *Spirogyra* species (Gupta and Rastogi, 2008), *Azadirachta indica* bark (King et al., 2008), *Rhizopus oryzae* (Bhainsa and DŠsouza, 2008), *Bacillus jeotgali* (Green-Ruiz et al., 2008) has been reported for efficient biosorption. The phenomenon of biosorption is an emerging technology, which is overpowering the existing methods as it does not leave any chemical sludge, highly selective, cost-effective, easy to operate and more efficient for treatment of wastewater in higher volumes (Hawari and Mulligan, 2006; Tunali et al., 2006; Volesky, 2007; Oh et al., 2009; Li et al., 2010; Oves et al., 2013; Fomina and Gadd, 2014; Aryal and Liakopoulou-Kyriakides, 2015).

Biosorption of heavy metals using bacterial, fungal and algal biomass (living or dead cells) has been found to be a potential alternative to conventional techniques. The process of biosorption involves physiochemical interactions between metal ions and several anionic ligands like carboxyl, phosphoryl, carbonyl and sulphydryl which are present on the surface of the biomass (Volesky, 1994; Volesky and Holan, 1995). The efficiency of the biosorption method depends on the type of metal ion being investigated to the type of biosorbent (Tunali et al., 2006). Biosorption is an interdisciplinary branch of science and technology anticipating the potential of low cost, highly efficient and waste minimizing approach. Therefore, from the above literature, it is well established that microorganisms can be isolated from various waste laden sources and utilized for the purpose of micro-bioremediation. Most of these reports were aimed at isolation of microbes from soil, wastewater, industrial waste, plant roots, and mining area. However, literature for the isolation of microbes from water hyacinth compost followed by their utilization for biosorption study is missing. In addition, there are limited reports available on the biosorption study with a live and dead form of biomass at the batch and continuous column mode. Hence, the present study was

focused on the isolation and identification of microbe during rotary drum composting of water hyacinth followed by its utilization in biosorption of heavy metals such as lead and cadmium. Physiochemical, biological and microbial study of rotary drum composting of water hyacinth was performed, bacteria were isolated from the compost, followed by their utilization in biosorption of heavy metals in batch and column mode.

1.2 Hypothesis

Composting of biological waste is mainly governed by the diversity of microorganisms working intermittently or in succession in order to carry out the biochemical reactions for their metabolism. The presence of certain desirable communities is reported to enhance the composting process. The rotary drum composting is comparatively a faster method of composting as compared to conventional pile (windrow) composting method (Kalamdhad and Kazmi, 2009). It has been found that the total content of metal has increased to a great amount in final compost (Singh and Kalamdhad, 2012). Therefore, it is hypothesized that a combination of robust microbial consortia is present in water hyacinth compost, surviving in unfavorable and variable conditions of composting. Efficient microbes can be isolated from the water hyacinth compost which can eventually be utilized for the purpose of biosorption of heavy metals, favoring the goals of micro-bioremediation and waste and wastewater treatment.

1.3 Objectives

The main objective of the study was to isolate and identify the microbial community from rotary drum compost of water hyacinth, followed by the application of isolated microbes in biosorption process.The scope of the present study was limited to:

- Microbial population, stability and maturity analysis of rotary drum composting of water hyacinth mixed with cow dung and sawdust in different proportions.
- Enumeration of microbial communities from the best proportion of compost, followed by the isolation and identification of microbes from the rotary drum compost of water hyacinth.
- Batch biosorption study of lead and cadmium by live and dead cells of microbe isolated from the compost of water hyacinth.
- Biosorption study of lead in column mode by bacteria isolated from the compost of water hyacinth.

1.4 Need of the Study

Composting is a rigorous process of aerobic degradation of organic waste due to the metabolic activities of microorganisms, therefore it serves for the abundance of metabolically active microbes (Hassen et al., 2001; Ryckeboer et al., 2003; Anastasi et al., 2005; Park et al., 2005). Literatures are documented regarding the isolation of microbes from soil, wastewater, and industrial waste followed by their application in removal of heavy metals. Water hyacinth composting is a rigorous process of organic matter degradation. As the compost is enriched with huge microbial community despite the presence of heavy metals and robust conditions, the microbes present in it are anticipated of an efficient application in heavy metal removal.

Biosorption is becoming an essential component of the integrated approach towards the treatment of aqueous effluents. Many biological origin substrates including food wastes, agricultural waste, natural residues, cellulose, chitosan etc. have been investigated for the metal removal by biosorption process. Microbial biomass (bacteria, actinomycetes, fungi, algae, yeasts, and microalgae) were found as the most cost effective, economical, and efficient biosorbents to sequester the heavy metals by biosorption. Heavy metals such as Zn, Cd, Pb, Cr, Cu, Hg, Co, As, and Ni have been frequently investigated in the context of their removal by microbial biosorption.

The conventionally practiced methods of heavy metal removal including precipitation, electrochemical treatment, ion exchange, solvent extraction, reverse osmosis, and adsorption may end up with environmental problems because of the toxic secondary sludge production and also they are expensive and ineffective in treating very low concentration of heavy metals (Volesky, 2007; Fomina and Gadd, 2014). Biosorption is an emerging technology in the field of heavy metals removal and wastewater treatment. Biomasses originating from different wastes have been exploited for their inherent property of interacting with heavy metals with respect to biosorption process (Volesky, 1994; Anastasi et al., 2005; del Carmen Vargas-García et al., 2012).

1.5 Scope of the Thesis

To achieve the above mentioned objectives, laboratory experiments as well on-site studies were performed alternatively. In the first step, the experimental set-up for rotary drum composting of water hyacinth was organized, its performance was determined by the physiochemical analysis, biological analysis, and microbial analysis of the compost. The learning of the protocols and operation of instruments was performed simultaneously. Secondly, the best waste combination of water hyacinth compost was used for the isolation and identi-

fication of microbes as well as the 16S metagenomics profile. This was followed by the treatment and removal studies of heavy metals such as lead and cadmium by the isolated microbial species. Both live (unpre-treated) and dead (pre-treated) microbial biomasses were investigated for the biosorption studies. Batch as well column mode operation was investigated for metal removal by the dried microbial biomass.

1.6 Thesis Organization

The thesis has been organized in following chapters:

- Chapter 1 gives a brief introduction to the problems of water hyacinth, composting, isolation of microbes from various waste sources and their utilization in heavy metal removal, biosorption with microbial biomasses, objectives, the need of the study and scope of the thesis.
- Chapter 2 gives a detailed literature review of water hyacinth and heavy metal problems, isolation and characterization of microbes from different waste sources and biosorption studies with isolated microbial strains.
- Chapter 3 deals with the collection and initial characterization of the raw materials such as water hyacinth, cow dung, and sawdust. Experimental design of phase 1, 2, 3 and 4 is given in flowchart. A detailed procedure of the experimental design is provided.
- Chapter 4 deals with the microbial succession during rotary drum composting through 16S rRNA gene sequence method and the isolation and characterization of bacteria from rotary drum compost of water hyacinth.
- Chapter 5 gives the details of biosorption study of lead and cadmium with the isolated bacterial strain in the live and dried form in the batch operation mode. Removal of lead by dried bacterial biomass in continuous column mode.
- Chapter 6 includes the conclusions and future recommendations of the thesis.

"Water and air, the two essential fluids on which all life depends, have become global garbage cans."

Jacques-Yves Cousteau

2

Literature Review

This chapter covers the detailed review of the literature available related to the work. A brief summary of the information has been presented in this chapter.

2.1 Water Hyacinth

The water hyacinth (*Eichhornia crassipes*) is noxious plant and considered as world's worst free floating aquatic weed (Holm et al., 1991). A native of Amazon basin in South America, it was introduced to various parts of the world as an ornamental plant, now it has become a problematic weed in different parts of the world including USA, Africa, Europe, Southeast Asia and Australia. In India, water hyacinth covers 23.2% wetland area of the northeast region (Abbasi, 1998; Husain, 2003; Shanab et al., 2010). The plant is perennial aquatic weed belonging to family *Pontederiaceae*. It is usually 40 cm high but it can mature up to the height of 1 m. The stem and leaves have air-filled tissue which gives buoyancy to the plant causing the leaves to float above water surface with long spongy stalks, the inflorescences bear lily-like flowers (Herfjord et al., 1994). Water hyacinth reproduces sexually and asexually by seeds and vegetative reproduction (budding) respectively (Malik, 2007). This inherent property of fast growth results into formation of dense mats up to 2 m thick. Eventually, it causes major problems in the whole area such as reduction of fishes due to obstruction of light and oxygen, obstruction of shipping routes due to dense mat formation, water loss due to higher evaporation and interference with irrigational activities (Gunnarsson and Petersen, 2007; SushilKumar, 2011). It degrades water quality by blocking photosynthesis, takes water nutrients, resultantly it causes the eutrophication of aquatic system. Also, the fast growing tendency of water hyacinth restricts the growth of other plants causing loss of wetlands due to decline in quality and quantity of water(Dhal et al., 2012). It is now considered as a serious threat to biodiversity. Water hyacinth has

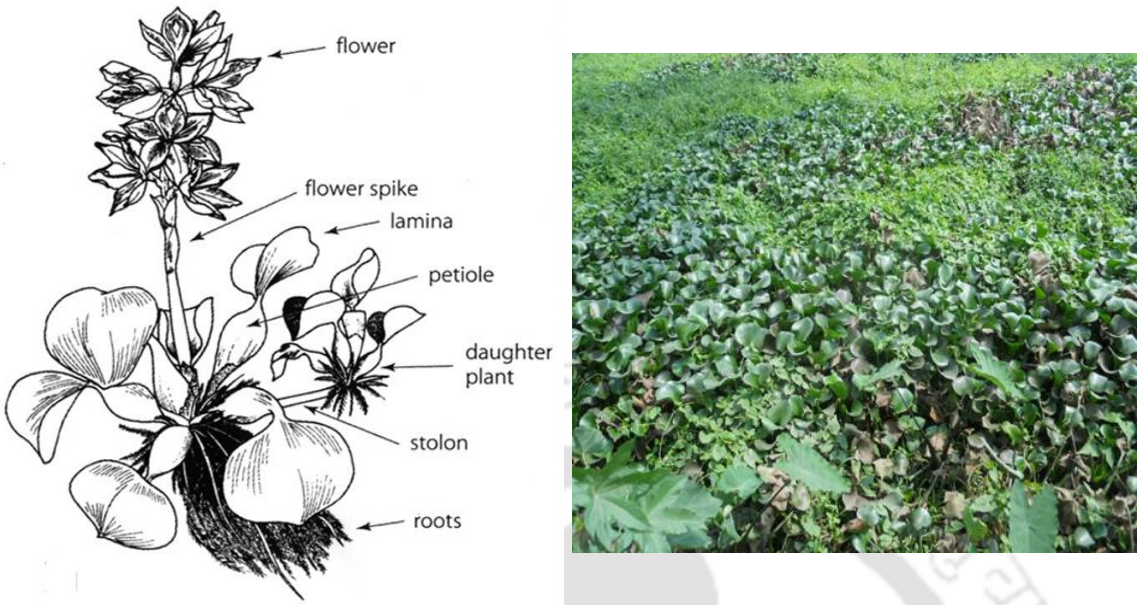


Figure 2.1: Water hyacinth plant

been utilized in many ways such as substitute for animal fodder (Tag El-Din, 1992), feed for fermentation process and biogas production (Singhal and Rai, 2003), as a raw material for pulp and paper industries (Goswami and Saikia, 1994), in wastewater treatment plant and biogas production (Malik, 2007). As water hyacinth can absorb nutrients in excess for its fast growth it has been utilized for sewage treatment (Moran, 2006). Water hyacinth (Figure 2.1) has an ability to grow in water polluted with heavy metals due to its capacity to accumulate metal ions, this has raised attention for its exploitation in phytoremediation for wastewater treatment (Malik, 2007). The composting and vermicomposting of water hyacinth are an advantageous alternative treatment as well as methods of utilization of water hyacinth (Gajalakshmi et al., 2001; Gupta et al., 2007; Singh and Kalamdhad, 2013a,b).

2.2 Composting

Composting is a process of decomposition of organic matter as a result of the thermophilic temperature developed due the metabolic activities of microorganisms (bacteria, actinomycetes and fungi) in presence of oxygen forming a stable pathogen free end product i.e. compost which can be beneficially applied to land (Roger, 1993; Bhardwaj, 1995; Abbasi and Ramasamy, 1999). In the process of composting, organic matter is broken down to produce carbon dioxide, heat, water, and the stable organic end product i.e. compost. The optimal conditions of composting can be distinguished on the basis of the temperature variations. The composting process proceeds through three phases such as (a) the mesophilic,

or moderate-temperature phase, in which microorganisms acclimatize themselves into the surrounding environment (b) the thermophilic, or the highest temperature phase, which causes the highest degradation of the organic matter into stable pathogen free end product, it can last from a few days to several months, and finally, (c) Maturation and cooling phase, the temperature reduces down up to mesophilic and ambient level, it can last up to several months. The aerobic degradation of organic waste can be expressed by the simplified chemical formula (Eq. 2.1)

$$C_6H_{10}O_4 + 6.5\,O_2 = (C_6H_{10}O_4)_{a-1} + 6\,CO_2 + 5\,H_2O \tag{2.1}$$

The (Eq. 2.1) is an exothermic reaction which generates 616 Kcal of heat per mole of the organic matters degraded, assuming 230 kcal/mol is the heat of formation of the organic matter. Thus, for degradation of a glucose molecule, -673 Kcal/mole of heat is generated followed by production of carbon dioxide and water (Themelis and Kim, 2002). Thus, for water hyacinth, composting is a better alternative in producing compost having valuable application for soil as compared with other treatment methods of water hyacinths. Generally composting is divided into two types, as the open systems and the in-vessel systems. An open system of composting includes windrow or pile composting, they are also called as non-reactor composting, being the most preliminary type of composting procedure (Roger, 1993). The in-vessel system of composting comprises of the tunnel systems, and the rotary drum composting (Gajalakshmi and Abbasi, 2008). The rotary drum composting is one of the promising decentralized composting techniques. It provides mixing and aeration of the waste organic matter, to produce a consistent and uniform compost of water hyacinth (Kalamdhad and Kazmi, 2009). The different phases of the composting period are predominated by varied microbial communities. At the mesophilic phase, microorganisms have the capability to rapidly break down the soluble, readily degradable compounds. A large variety of mesophilic and thermophilic microorganisms have been reported in composting of municipal and industrial solid waste.

2.3 Microbiology of Composting

Composting is a microbiological process of aerobic degradation of the heterogeneous organic matter by the metabolic activities of the successive mixed microbial population. The populations and communities of microorganism vary continuously, which makes the description and differentiation of microorganisms participating in the composting process difficult, microbial populations change rapidly as a function of the surrounding environmental conditions such as temperature, nutrient availability, oxygen availability, water, and

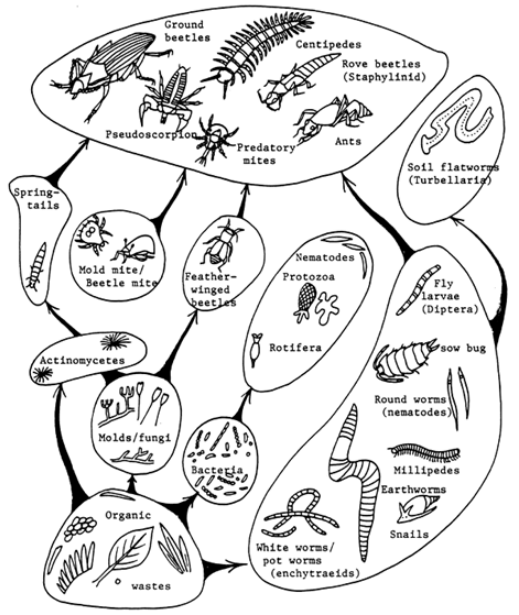


Figure 2.2: Food web of the compost pile ((National Research Council, 1981))

pH in the process of composting. These conditions are responsible for microbial survival and their activities, additionally different types of microorganisms act at different stages of composting. It is because of the metabolic activities of these microbes in the composting environment, the temperature fluctuations take place (Bhatia et al., 2012, 2013). The food web of composting process (Figure 2.2) explains that all other organisms also exist in compost but the major role of degradation is played by the microorganisms. Usually, the secondary level consumers appear during maturation stage. While tertiary consumers appear during final stages of composting or near end of composting. The microbes have known to do the degradation hence are studied extensively to understand the process of composting. Every microbe has peculiar characteristics features and can be distinguished. These microbes require specific environmental conditions and sufficient substrate to grow and multiply in number. A large variety of mesophilic and thermophilic microorganisms have been reported in composting of municipal and industrial solid waste. Specific type of microorganisms i.e. bacteria (*Bacillus* sp., *Thermus* sp.), fungi (*Cladosporium*, *Alternaria*, *Verticillium*, *Aspergillus*, *Eurotium*, *Penicillium*, *Trichoderma*, *Mucor*, and *Rhizopus* sp.) and actinomycetes are involved in breaking of the organic material, also they have been reported as primary decomposers in composting (Strom, 1985; Beffa et al., 1996; Pedro et al., 2003).

2.3.1 Bacteria

Bacteria play an important role in organic matter degradation during the composting process, as they comprise of 80-90% of the billions of microorganisms present in a gram of compost material (Trautmann and Olynciw, 2012). Bacteria have more survival capability as they are capable of utilizing organic materials for their metabolic actions, their broad range of enzymes make them capable of feeding on a wide range of organic matter (Golueke, 1992; Epstein, 1997; Raut et al., 2008; Liu et al., 2011). Bacteria majorly participate in the mesophilic and thermophilic stages of composting. Bacteria targets the initial decomposition of the robust and hard to degrade organic matter in the initial stage of composting, also their action is majorly responsible for the temperature rise in composting process to a thermophilic phase (above 40°C). In composting process, the bacterial species present commonly are such as *Bacillus, Pseudomonas, Enterobacter*, and *Acinetobacter* (Strom, 1985; Bhatia et al., 2013).

In the mesophilic and thermophilic stages of composting bacterial species of *Bacillus badius, Bacillus pumilus, Bacillus sphaericus*, and *Bacillus thuringiensis* dominate the process (Ryckeboer et al., 2003). High surface to volume ratio, a broad range of organic matter degrading enzymes and less generation time make bacteria more capable of surviving the rapid changes of substrate availability and other surrounding conditions during the composting process. Consequently, bacteria are major role players in initial decomposition and temperature rise during the composting procedure (Ryckeboer et al., 2003). There are reports which state that CO_2 evolution rate of mesophilic bacteria such as *Bacillus* sp. and *Azotobacter* sp. were highest in the initial phase of composting (Temperature less than 40 °C), they have caused major degradation of the mixed raw organic material (Nakasaki et al., 1985). Although composting is an aerobic process, facultative anaerobic bacterial species of *Bacillus* and *Thermoactinomyces* genera have been reported in rotary drum composting of biodegradable organic wastes (Bhatia et al., 2013). It has been observed that at the beginning of composting process gram positive rod shaped bacteria are dominantly present, while at the end of composting period gram-negative bacilli shaped bacteria dominate (Bhatia et al., 2012). Relevant changes in microbial population occur with the availability and complexity of the organic material present as well as the temperature variation plays an important role in the activity and type of microbial species of the composting cycle.

2.3.2 Actinomycetes

Actinomycetes resemble the characteristics of bacteria as well as fungi, they lack nuclei like bacteria and grows into mycelium like fungi and with no chitin and cellulose in the cell

wall. The earthy smell of soil is because of the production of sesquiterpenoid compounds i.e. geosmine by the actinomycetes, and are capable of degrading complex organic material such as lignin and cellulose, they also possess enzymes to break down the tough woody debris, bark and other cellulolytic material. Actinomycetes develop slowly as compared to most bacteria and fungi and can inhibit microbial growth by producing antibiotics, lytic enzymes and can also parasitism (Ryckeboer et al., 2003).

Moreover, they can tolerate higher pH than fungi, the optimum pH range of growth is between 7 and 8 and they can also develop spores in adverse surrounding conditions. The optimum temperature for growth of actinomycetes is between 25- 30°C, except some species which are resistant to higher temperatures above 60°C (Nakasaki et al., 1985). However, maximum lignin degradation by actinomycetes along with thermophilic fungi takes place between the temperature range of 40-50°C (Gajalakshmi and Abbasi, 2008). These microorganisms are not only present majorly during thermophilic stage of composting but also during maturation and curing stage. A white film of actinomycetes species such as *Thermoactinomyces* sp. and *Streptomyces* sp. have been reported after the maturation of compost (Strom, 1985).

2.3.3 Fungi

Fungi comprise of molds and yeasts and are majorly causing the degradation of complex plant polymers such as lignin. Fungi are the dominant active degraders of fresh organic matter in the mesophilic and thermophilic stage of composting within the temperature range of 20-40 °C, a pH range of 4-5 supports the growth of fungi and yeasts (Ryckeboer et al., 2003). Their tendency of spreading into many cells and filaments make them capable of degrading organic matter that is acidic or low in nitrogen composition, which is difficult for bacteria to degrade. With further degradation, ammonification, and increase in pH, the fungi community reduces. In composting process, the majorly found fungal species are such as *Acremonium* sp. *Actinomucor* sp., *Aspergillus*sp., *Candida* sp. *Fusarium*, *Penicillium*, and *Graphium* (Ryckeboer et al., 2003; del Carmen Vargas-García et al., 2012).

The high temperature is unfavorable for fungal growth, the optimal temperature for growth of thermophilic fungi ranges from 45-50°C. Above 65°C, no fungal growth has been observed, however, thermophilic fungi have been observed to have cellulolytic or lignolytic activity at higher temperatures (Nakasaki et al., 1985; Hellmann et al., 1997). Apart from temperature, other factors such as pH and availability of carbon and nitrogen, most fungi prefer acidic conditions and high nitrogen content, except the wood rot fungi which requires low nitrogen (Dix and Webster, 1995). Usually, fungi grow in form of unseen filaments as well as colonies on the compost surface.

2.4 Metal-Microbe Interactions

Many microorganisms are documented to have resistance to metals in various surroundings such as water, soil, and industrial waste (Bruins et al., 2000). There is a specificity of microbes with different types of metals which is encoded as genetic determinants on the genes located on chromosomes and plasmids. Some metals i.e. Co, Cu, and Ni serve as micronutrients for bacteria adjusting its functioning for influx and efflux of metals (Figure 2.3). Micronutrients are used for a metabolic process such as redox reactions to stabilize molecules through electrostatic interactions, as components of various enzymes and for osmotic pressure regulation (Vargas et al., 1997). Efflux of the heavy metals by bacterial biochemical mechanism has been studied at the molecular level (Bruins et al., 2000).

Common mechanisms include the membrane bound proteins, membrane transport systems, ATPases, P-type ATPases, proton antiporter systems (Cervantes, 1991; Vargas et al., 1997; Bruins et al., 2000). Most of the metals are toxic to the microbial mechanism, they may be essential but in small quantities. At higher quantity level they may damage the cytoplasm, cellular components, enzymes and also the DNA. Microorganisms have adapted themselves by various mechanisms including permeability, Intra- and extra-cellular sequestration, transport efflux pumps, enzymatic intervention, and loss of sensitivity of cellular targets to metal ions (Bruins et al., 2000). The most common terms are biosorption, bioaccumulation, bioleaching, bioprecipitation, biooxidation, bioreduction, bioprecipitation, biofloatation, bioflocculation, etc. The availability of heavy metals decides the activity and response of the microbes.

2.4.1 Mechanisms of Metal-Microbe Interaction

Microbes survive the heavy metal concentration due to the intrinsic or induced mechanisms, additionally, the environmental factors also impact the microbial growth. Microbes can cope up with the metal toxicity by tolerance due to its intrinsic properties, while the resistance of microbes occurs by the action and mechanism of detoxification (Iyer et al., 2005). More is the complexity of the microorganism's structure the more will be the ways of metal to be captured by the cell. Mechanism of microbial interaction can be broadly classified into two types as:

(a) Metabolism-dependent mechanism: This is the active mode of interaction between the microbe and metal. It includes the redox mechanism, sequestering, and ion transport. Microbes can use metals or metalloids as electron donors or acceptors in order to maintain their metabolism. The microbial oxidation and reduction occurs as a consequence of the metabolic activities with the metals and metalloids. Different metal forms such as oxidiz-

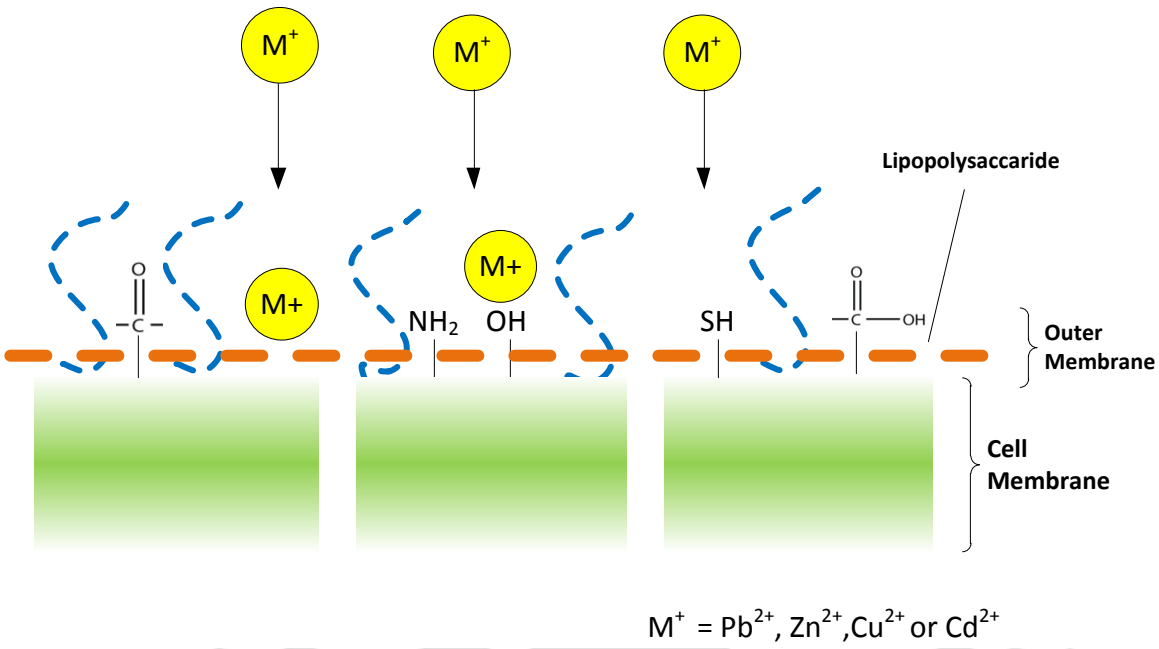


Figure 2.3: Metal and microbe interaction schematic diagram

able metals may provide energy demands of a microorganism eg. *Eubacteria*, anaerobic and hydrogen-oxidizing autotrophic bacteria. Also these enzymatic microbial interactions can lead to microbial detoxification (Ehrlich, 1997; Ahluwalia and Goyal, 2007; Gadd, 2009). Many microorganisms form biofilms and exo-polysaccharides which are capable of binding metals in natural conditions (Gadd, 2009). The interaction of the living cells with heavy metals generally incorporates the intercellular accumulation as well as surface (Ledin, 2000).

(b) Metabolism-independent mechanism: This is a surface phenomenon which involves interaction of the dead cells with the heavy metals on the cell surface. The bacterial biomass acts as a biosorbent and the heavy metal as a sorbate. As biosorption is a surface phenomenon in most biosorption processes the metal is recovered from the cell surface. Biosorption can widely be described as both active and passive phenomenon describing the fundamentals of interactions which include a sorbate with the biological material (Iyer et al., 2005). Mechanisms of biosorption have been studied and reported to be adsorption, ion exchange, complexation, precipitation, and crystallization (Garnham et al., 1992; Barakat, 2011). Various functional groups form the structural components on the biosorbent surface which include carboxyl, phosphate, hydroxyl, amino, thiol etc. Various mechanisms involved in biosorption act according to the availability of surrounding factors and nature of biosorbent (Garnham et al., 1992; Fomina and Gadd, 2014). Metal biosorption is physiochemical in nature, rapid and can be recovered back, in case of precipitation metal can be present on the surface of biological adsorbent as well as in the reaction solution

(Veglio and Beolchini, 1997; Volesky, 2007; Fomina and Gadd, 2014). On the other hand, some fraction of metal is retained as they are immobile. Binding of metals onto the bacteria can be affected by the metabolic interactions of bacteria resulting into the change in environmental fate of that metal through mechanisms such as reduction, oxidation, and precipitation reactions (Veglio and Beolchini, 1997; Bailey et al., 1999; Lesmana et al., 2009). The adsorption of metal holds an important position in the bioavailability of metals (Yu et al., 2000; Chojnacka et al., 2004; Saeed et al., 2005; Sharma and Bhattacharyya, 2005; Yang, 2005). Different kinds of the intrinsic or induced mechanisms can be involved in the accumulation of metals by microorganisms e.g. adsorption, precipitation, complexation, extracellular polymers and active transport into the cell (Ledin, 2000; Gadd, 2009).

However, the metal resistant biosorbing bacteria are used for various metal removal purposes. Biosorption of heavy metals using bacterial, fungal, and algal biomass (living, dead cells, and their derivatives) has been found as a potential alternative to the conventional techniques, since it does not produce secondary sludge, has higher selectivity, more efficient, easy to operate and cost-effective for treating large volumes of water and wastewater (Andres et al., 2003; Tunali et al., 2006; Li et al., 2010). Microbes possessing the ability to affect the state of metals can be exploited for bioremediation purpose (Xu et al., 2012; Prasad et al., 2013; Fomina and Gadd, 2014). For the purpose of biosorption, different robust microbes have been isolated from various waste sources such as wastewater, soil, sewage sludge, industrial effluent, and compost of horticulture and municipal wastes. Bacteria are diversified in different ecological habitats. They have valuable uses in environmental microbiology. Numerous microbes have been isolated from different waste sources for their exploitation in varied microbe-based technologies. Contaminated regions such as industrial waste, wastewater, soil, municipal waste and sewage sludge have been used as source for the isolation of the most robust microbes (Pal and Paul, 2004; Congeevaram et al., 2007; Bahig et al., 2008; Jiang et al., 2008; Shoeb et al., 2009; Çolak et al., 2011; Bautista-Hernández et al., 2012). Different species of *Pseudomonas, Bacillus, Phanerochaete, Tricholoma, Microbacterium, Micrococcus, Firmicutes, Actinobacteria, Proteobacteria, Serratia* sp. and *Atrhobacter* are reported to reduce Pb, Cr, Zn, Cd, Cu, Ni, and Cd (Congeevaram et al., 2007; Zhou et al., 2007; Mishra and Doble, 2008; Choudhary and Sar, 2009; Li et al., 2010; Çolak et al., 2011; Srivastava and Thakur, 2012; Ghosh and Thakur, 2016).

2.5 Isolation of Microorganisms from Waste Sources

A number of heavy metal resistant microbes have been isolated from various waste sources. As wastes are rich source of heavy metal contamination it can serve to comprise of consortia of heavy metal resistant microbes. A number of heavy metal resistant microbes

have been isolated from different sources of wastes i.e. waste water (Jose et al., 2011), soil (Jansen et al., 1994; Hassen et al., 1998; Pal and Paul, 2004; Sheng et al., 2004; Bahig et al., 2008; Shoeb et al., 2009; Sun et al., 2010; Stanbrough et al., 2013), sewage sludge and industrial wastes (Belimov et al., 2005; Mishra and Doble, 2008) as mentioned in Table 2.1.

2.5.1 Industrial Waste and Wastewater

Chromium tolerant microorganisms had been isolated from the dry solid waste and the liquid effluent of the industries and were tested for their chromium biosorption capacity. Taxonomic groups such as *Firmicutes, Proteobacteria, Actinobacteria*, and *Bacteroidetes* were found to be prevalent in the isolates (Mishra and Doble, 2008). *Pseudomonas* sp. and *Bacillus* strains were isolated from the wastes generated by the mines of uranium and were examined for their potential in removing heavy metals including uranium, they were found to be multi-metal resistant strains (Selenska-Pobell et al., 1999; Schmidt et al., 2004; Pollmann et al., 2006). *Pseudomonas aeruginosa* was isolated from wastewater of the Tunis city, and was found to be the most important isolate, thus it was selected as bioindicator of toxicity (Bai and Abraham, 2003).

Soils near the electroplating industry were taken as source for isolation of Cr(VI) and Ni(II) resistant fungi and bacteria. Thereby, these isolates were characterized for their evaluation of heavy metal removal from industrial wastewaters (Congeevaram et al., 2007). Nine novel chromium tolerant microorganism were isolated from the wastes of tannery, electroplating and electronic industries containing chromium contamination, they were tested for chromium removal and were found to tolerate chromium concentration up to 700 mg/L. 16S rRNA results of two most active organisms indicated the presence of *Bacillus marisflavi* and *Arthrobacter* sp. (Mishra and Doble, 2008). Strain GESQA002 was isolated from the soils near the automobile and welding workshops contaminated with mutilple heavy metals. It depicted multiple heavy metal resistance against Cd, Cu and Ni. Additionally, it also depicted resistance against antibiotics such as Kanamycin and streptomycin also (Shoeb et al., 2009).

2.5.2 Soil

Soil is a good source of various kinds of heavy metals and microorganisms which tend to develop resistance against the surrounding heavy metals. As heavy metals can affect the growth, morphology, and metabolism of soil microorganisms and consequently lose soil fertility (Bragato et al., 1998). The biochemical mechanisms provide the microbe a

Table 2.1: List of microbes isolated from various sources.

Microbes	**Metals**	**Source**	**References**
Pseudomonas aeruginosa	Cd, Cr, and Ni	Wastewater	(Hassen et al., 1998)
Pseudomonas sp. and *Bacillus* sp.	U	Uranium mine waste	(Selenska-Pobell et al., 1999; Schmidt et al., 2004; Pollmann et al., 2006)
Ralstonia metallidurans	Zn	Zinc decantation tank	(Mergeay et al., 2003)
Bacillus sphaericus	Cr	Soil of Andaman Islands	(Pal and Paul, 2004)
Micrococcus sp. and *Aspergillus* sp.	Cr and Ni	Electroplating industry waste	(Sheng et al., 2004; Congeevaram et al., 2007)
Pseudomonas fluorescens and *Microbacterium* sp.	Pb	Plant roots of Pb contaminated soil	(Sheng et al., 2008)
Burkholderia sp.	Pb and Cd	Heavy metal-contaminated soils	(Jiang et al., 2008)
Bacteria CCNWRS33-2	Cu, Cd, Zn and Pb.	Root nodule of *Lespedeza cuneata*	(Wei et al., 2009)
Acidobacteria, Actinobacteria, Bacteroidetes, Gemmatimonadetes	Ni, Cd, Zn, Co, and Cd	Soil samples near farmland	(Karelová et al., 2011)
B. arsenicus, B. pumilus, B. arsenicus, B. indicus, B. clausii, P. maritimus and *Staphylococcus pasteuri*	As, Hg, Co, Cd, Pb, and Se.	Soil of Eloor (India)	(Jose et al., 2011)
Graphium,putredinis, Fusarium solani, Fusarium sp. and *Penicillium chrysogenum*	Cd, Cr, Ni, Pb, and Zn	Horticulture waste, sewage sludge and municipal solid waste compost	(del Carmen Vargas-García et al., 2012)
Achromobacter sp. strain AO22	Cd and Zn	Soil near *Crotolaria juncea*	(Stanbrough et al., 2013)

capability to endure the heavy metal intoxication. Therefore, soil also serves as one among the rich sources of heavy metal resistant microbes. Large group of chromium-resistant bacteria were isolated from the serpentine soils of Andaman Islands, India, serpentine soils are contaminated due to chromium percolation. All the isolates depicted varied degree of chromium removal notifying the role of inherent genetic factors responsible for metal resistance. *Bacillus sphaericus* was found to be the most resistant bacteria among all the bacterial isolates (Pal and Paul, 2004).

Endophytic bacteria were isolated from the roots of rape plant grown in heavy metal-contaminated soils and were later characterized for their efficiency of lead removal. Isolates were identified to be *Pseudomonas fluorescens* and *Microbacterium* sp. by the 16S rDNA gene sequence analysis (Sheng et al., 2004). *Burkholderia* sp. isolate was also evaluated for promoting plant growth, Pb and Cd uptakes by the plants from the metal contaminated soil in the pot experiment (Jiang et al., 2008). Bacterial clones belonging to four broad taxonomic groups *Acidobacteria, Actinobacteria, Bacteroidetes* and *Gemmatimonadetes* were isolated from the soil near farmland, they were found to be resistant to metals such as, Ni, Cd, Zn, Co, and Cd. Among the isolated colonies, bacteria belonging to the group

Actinobacetria were prevalent, genes present in them were found to be related to the metal resistance, this could be of great potential for being used in genetic modification, cloning, and transformation for the bioremediation purpose (Karelová et al., 2011). Resistance of metals such as Zn, Co, Ni, and Cu was observed among the bacterial isolates retrieved from soils of highly polluted areas of Kerala in India such as Eloor, Vypin, and Munambam. Among the 24 isolates *Bacillus arsenicus, Bacillus pumilus, Bacillus arsenicus, Bacillus indicus, Bacillus clausii, Planococcus maritimes* and *Staphylococcus pasteuri* depicted high resistance against metals such as As, Hg, Co, Cd, Pb, and Se (Jose et al., 2011).

Bacterial strain *Achromobacter* sp. AO22 was isolated from the lead contaminated regions of Australia. This soil microbe was tested for the growth of plant sunn hemp (*Crotolaria juncea*) in the presence of Cd and Zn. Growth of roots and shoots of the plant were observed due to the inherent genetic mechanism of the bacteria enabling the efflux of metals and preventing metal uptake by the plant. This study depicted that soil microbe such as AO22 can be used for safer plantation curbing the ill effects of heavy metals on plants (Stanbrough et al., 2013).

Biosorption study for the *Bacillus* sp. previously isolated from soil polluted with heavy metals was performed. Both Pb and Cu ions removal capacity was checked in the binary system in the aqueous solutions. Single metal and competitive metal removal was estimated in the bacterial isolate, therefore the bacterium is inexpensive, easy to obtain and culture with maximum biosorption capacity of 92.3 mg/g at 250 mg/L of Pb and 16.3 mg/g at 200 mg/L of Cu (Tunali et al., 2006).

Bacillus cereus and *Bacillus pumilus* strains were isolated from soil located in the Kütahya (Emet) region of Turkey. Dried biomass of both the isolates was used as metal adsorbents for the removal of Pb in batch, fixed-bed, and the column mode operation. The maximum uptake of Pb by *B. Cereus* and *B. pumilus* bacteria were found to be 23.3 and 29.6 mg/g respectively. Higher biosorption capacity was observed to be in fed-batch system than the batch system (Çolak et al., 2011).

Bacterial strain identified as *Delftia tsuruhatensis* was isolated from the dumps of mine and was used for biosorption of Zn and Pb. Dried bacterial biomass (0.015 g) was inoculated for the removal study, the maximum biosorption was reported to be 0.22 and 0.21 mmol/g for Pb and Zn respectively (Bautista-Hernández et al., 2012).

2.5.3 Plant Roots

Plants growing in heavy metal contaminated sites absorb the metals via its roots. Microorganisms thriving in the soil with the plant roots tend to develop resistance against the surrounding heavy metals. Therefore, the plant roots also serve as a reserve for the heavy metal resistant microbes. Numerous microbes had been isolated from roots of different plants growing in contaminated region. Roots of Indian mustard *Brassica juncea L. Czern.* were used as a source for isolation of bacterial strains which were then found to be tolerant to Cd. The plant was grown in the soil supplemented with sewage sludge and mining waste highly contaminated with Cd. Eleven different strains were finally isolated. In addition to Cd, bacteria also showed increased tolerance to other metals such as Zn, Cu, Ni, and Co. The isolated strains were identified to be *Variovorax paradoxus, Rhodococcus sp*., and *Flavobacterium* sp., the strains were capable of supporting growth of *B. juncea* seedlings even in the presence of Cd. These isolated bacteria can be utilised as soil inoculants for improving growth of plants which get stunted by the presence of heavy metals (Belimov et al., 2005).

Heavy metal resistant bacterial strain named as CCNWRS33-2 was isolated from the root nodule of *Lespedeza cuneate*. The plant was grown in the contaminated regions of gold mine dumps in China. The isolated strain was tested and found to be highly resistant to metals such as Cu, Cd, Zn, and Pb. The heavy metal stress could not affect the mean specific growth rate of bacteria, in the presence of metal also the bacteria depicted substantial growth with bioaccumulation. The phylogenetic study of bacterial isolate by the 16S rRNA gene sequence technology depicted that CCNWRS33-2 had 98.9% similarity to the nitrogen-fixing legume symbiont i.e. rhizobium *Agrobactrium tumefaciens* LMG196 (Wei et al., 2009).

2.5.4 Compost

Composting is a rigorous process of aerobic degradation of organic waste by the metabolic activities of the microorganisms, therefore it serves for the abundance of metabolically active microbes (Hassen et al., 2001; Ryckeboer et al., 2003; Anastasi et al., 2005; Bhatia et al., 2013; Pan and Sen, 2013). These microbes are dependent on the surrounding conditions such as the temperature, availability of organic waste, pH, species diversity and availability of heavy metals. A Large variety of mesophilic, thermotolerant microorganisms have been reported in composting of different types of waste materials, it includes bacteria, actinomycetes, streptomycetes and fungi (Hassen et al., 2001; Ryckeboer et al., 2003). Qualitative and quantitative composition of mycoflora of compost and vermicompost of pant debris and animal wastes was performed by the isolation and identification of fungi from the compost. An abundance of *Chrysosporium* sp., *Penicillium* sp., and *Aspergillus* sp. was observed in the study (Anastasi et al., 2005). The heavy metals presence raises serious concern about the adverse environmental impact of compost application to the agricultural lands (Singh and Kalamdhad, 2012). Apparently the compost also serves as reservoir of heavy metal resistant bacteria. These bacteria are present in the metal loaded condition of the compost and yet degrading the organic matter. They are robust and can survive in high metal concentration. A group of 51 microorganisms was obtained from the compost of horticulture waste, sewage sludge and municipal solid waste and most of them depicted the capability to tolerate heavy metals such as Cd, Cr, Ni, Pb, and Zn (del Carmen Vargas-García et al., 2012). Bacterial diversity in full scale rotary drum compost of organic waste was observed to be mainly total heterotrophic bacteria such as *Bacillus* sp., *Pseudomonas* sp., and *Enterobacter* sp. (Bhatia et al., 2013).

A fungal strain XJ-1, phylogenetically related to *Penicillium chrysogenum* was isolated from compost of chicken manure. The fungal strain was investigated for its capacity to remove heavy metal such as Cd. The maximum adsorption capacity was found to be 100.41 mg/g (Xu et al., 2012).

2.6 Adsorption Study of the Bacterial Isolates

After being isolated from various heavy metal contaminated regions the bacterial isolates were examined for their removal efficiency of different metals. (Table 2.2) summarizes the utilisation of bacterial biomass for heavy metal removal in various ways. Biosorption studies with the live (non-pretreated) and dead biomass had been done. Bacterial biomasses from same or different genus were used for various studies of heavy metal removal in same or different conditions. The effects of certain physiochemical factors such as pH, initial

Table 2.2: Microbes and their metal removal efficiency

Microbe	Metal	Operating conditions				Removal efficiency/uptake	Reference
		pH	Temp	Contact time	Other information		
Streptoverticillium cinnamoneum	Pb, Zn	Pb=3.5-4.5 Zn=5-6	NA	30 min	Dried biomass	Pb(76%), Zn(96%)	(Puranik and Paknikar, 1997)
Citrobacter strain	Pb, Cd, and Zn	4.5,6,6.5	40°C	20 min	Dried biomass	90%	(Puranik and Paknikar, 1999)
Sargassum sp., *Padina* sp.	Pb, Cu, Cd, Zn, and Ni	Pb, Cu=5; Cd,Zn, Ni=5.5	NA	60 min	Dried biomass	90%	(Sheng et al., 2004)
Citrobacter freundii, Klebsiella pneumonia	Pb	4	25±2°C	100 min	Dried biomass	481.2 mg/L with 2g/ L	(Al-Garni, 2005)
Bacillus sp.	Pb and Cu	Pb=3; Cu=5	25°C	15 min (Pb); 30 min(Cu)	Dried biomass	92.27 ± 1.17 mg g-1; 16.25 ± 1.64 mg/g at 200 mg/L metal	(Tunali et al., 2006)
Bacillus cereus and*Bacillus pumilus*	Pb	6	25°C	5-100 min	1g/L of dried biomass	28.06 mg/g, 22.1 mg/g	(Çolak et al., 2011)
Delftia tsuruhatensis	Pb, Zn	NA	NA	20 min(Pb); 40 min(Zn)	0.015 g dried biomass	0.216 mmol/g (Pb); 0.207 mmol/g (Zn)	(Bautista-Hernández et al., 2012)
Graphium putredinis, Fusarium sp., *Penicillium chrysogenum*	Cd, Cr, Ni, Pb, Zn	NA	NA	NA	Living and dead biomass	95% (Pb), 80% (Zn), 70% (Cd, Cr, Ni)	(del Carmen Vargas-García et al., 2012)
Geobacillus thermodenitrificans, Geobacillus thermocatenulatus	Cu, Pb and Zn	Pb=4; Cu, Zn=5	NA	NA	Dry biomass 0.5 g/L	57 ± 4 mg/g (Cu), 18 ± 34 mg/g (Zn);	(Babák et al., 2013)
Bacillus thuringiensis	Cd, Cr, Cu, Pb and Ni	Ni, Cr= 7; Cd, Cu, Pb= 6	NA	30 min	Metal conc. = 25 mg/L	Ni(94%), Cu(91.8%), Cd(87%)	(Oves et al., 2013)

metal concentration, biosorbent dosage and contact time on biosorption wer assessed in relation to the heavy metal removal capacity. Mostly Pb, Cu, Zn, Cr, and Ni were a part of the investigation for metal removal by the biomass. Also for most of the metal ions the weak acidic conditions favoured maximum biosorption. This is because of the involvement of the acidic functional groups, carboxyl ions and amine groups. At higher pH metal hydroxides can be formed thus significantly reducing the amount of metal ions adsorbed at higher pH.

As the bacterial cellular composition varies with the genus the extent of biosorption also varies with the bacterial genus. Bacteria were isolated from various heavy metal contaminated locations, those were the robust and had developed resistance against the unfavourable conditions. This effect was apparently due to the production of significant amounts of extracellular organics in course of the metabolic activities of bacteria. The main mechanisms of metal resistance involved the adsorption by extracellular polysaccharides, sequestration of metals as insoluble phosphates, and efflux of ions outside the cell. The proportion removed by extracellular adsorption was different than that removed by intracellular accumulation. Thus, it signified that efficiency of living cells was different that of dead biomass. The degree of biosorption is attributed towards the metal ions concentration as well as the nature of biomass. The kinetics study of the bacterial biomass for biosorption gives an overview of its predictable nature and its applicability on large scale for the wastewater treatment technologies.

2.7 Lead and Cadmium in Environment

Heavy metals are defined as metals or metalloids present naturally in the earth, and became accumulated as a result of anthropogenic activities resulting into ecotoxicity (Duffus, 2002). Heavy metals are disposed from the outlet of various industries such as mining, smelting, metal plating, and ore processing which causes hazardous effects on humans, animals, and environment (Volesky and Holan, 1995; Duffus, 2002). At very low concentrations heavy metals are essential for metabolic activities of living organisms but can cause severe toxic effects on humans, plants and animals at higher concentrations (Lo et al., 1999). Mercury, cadmium, and lead called “the big three” have major impact on the environment among all the toxic heavy metals (Volesky, 1994).

2.7.1 Causes and Effects of Lead (Pb(II)) Contamination

Lead is present in the earth’s crust majorly as lead sulphide at a low level. The widespread prevalence of lead is a result of human activities such as industries, lead acid batteries, soldering, mining, paints, ammunition, smelting, pipes, use of leaded petrol (gasoline), jew-

ellery making, electronic waste and plastic stabilizers (WHO, 2011). Consequently, lead is the one of the most prevalent heavy metal contaminant on earth. The exposure modes of lead to the environment include contaminated air, water, soil, food, and consumer products.

Causes

• Industrial processes

The production of lead-acid batteries, plumbing materials and alloys use lead mainly. Additionally, lead is also used in paint industries, cable making, insecticides and ammunition. Occupational exposure is a common cause of lead poisoning in adults (Staudinger and Roth, 1998). Contact with materials painted with lead-containing paints through welding, cutting, grinding, construction, fabrication, electronic waste recycling, petrol etc. are significant sources of human exposure to lead.

• Food and smoking

Smoking of tobacco causes lead exposure. The people who are non-smokers are accustomed to lead exposure through either food intake or the materials used in storing and cooking food. The amount of lead present in the plants depends upon the concentration of lead in soil, mostly soils in the region of mining and smelting are contaminated with lead. Food colouring, contaminated spices, food and beverage cans made with lead soldering can also cause food contamination. Especially for storage of alcoholic drinks and acidic foods, storage in cans should be avoided. Also, usage of pottery having lead glazed ceramic causes intake of lead through food.

• Drinking water

Significant adverse effects are associated with usage of lead containing plumbing materials. Lead is barely present in tap water. However, water present in these pipes comes in contact with lead, especially the overnight or after longer duration storage will have higher concentration of lead. Acidic water dissolves most of the lead.

• Domestic sources

Lead contaminated paint on the household walls and dust are the main sources of exposure for infants and young children. Some toys made of lead containing plastic and paints, and also other traditional makeup (e.g. kohl).

• **Exposure routes of lead in environment and its effects**

The biogeochemical cycle in the environment influences the chemical and physical properties of lead to move throughout the ecosystem. The metal can enter into the different components of environment and affect them until reaching the equilibrium. Lead gets accumulated in different components of environment such as plants, ground, and water surface. Lead is the most ubiquitously spread heavy metal in the soil. It exerts adverse effects on the germination, physiology, morphology and metabolism of plants (Nagajyoti et al., 2010).

• **Effects on humans**

Lead is a cumulative general poison, infants and pregnant women being most susceptible to adverse health effects, it deteriorates the central nervous, kidney and the reproductive system (Sheng et al., 2004). Lead attacks the brain and central nervous system leading to coma. Children suffering from lead poisoning can have mental disorder. Other effects of lead exposure include anaemia, hypertension, immune dysfunction, renal failure, toxicity in reproductive organs (Staudinger and Roth, 1998; WHO, 2011).

• **Effects on soil**

Urban and agricultural soils are contaminated with lead due to mining, manufacturing, paint industries, pesticides, batteries, and industrial waste. It can result in heavy metal contamination of urban and agricultural soils. Lead in the soil can be ingested by children through hand-to-mouth activity, eating vegetables grown in the soil and from inhalation of re-suspended lead in the air (UNEP, 2010).

• **Permissible limits of lead**

According to world Health Organization (WHO) the permissible limits lead in drinking water is 10 μg/L and for air is 0.5 $\mu g/m^3$. The provisional tolerable weekly intake (PTWI) of lead should be 25 μg/kg of body weight for infants and children, considering that lead is a cumulative poison there should be no accumulation lead in body. The Indian standard for lead by Bureau of Indian Standard (BIS) in drinking water is 0.01 mg/L (BIS, 2009) and for air is 0.5 $\mu g/m^3$ (CPCB, 2009).

2.7.2 Causes and effects of Cadmium (Cd(II)) Contamination

Cadmium is present in earth's crust at a concentration of 0.1-0.5 ppm in common association with zinc, lead, and copper, it is also present naturally in the ocean waters with

average levels of 5-110 ng/L (Kirk, 2007). Common sources of heavy metal contamination are mining, industrial wastes, batteries, plastics, fertilizers, vehicle emissions, paints etc. (Waalkes, 2000; Kirkham, 2006; Oh et al., 2009). Exposure modes of Cadmium to the environment include contaminated air, water, soil, food, and consumer products.

Industrial processes: The production of NiCd batteries also causes cadmium deposition. Also the consumption of refined cadmium in pigments preparation for the plastics, enamels and ceramics deposits cadmium in household surroundings. Iron and steel production, cement production, alloying with metals such as lead, tin, and copper

Food and Smoking: Food contamination by cadmium can be caused by the cadmium polluted soil, or usage of cadmium-contaminated water for irrigation.Cigarette smoking increases risk of cadmium exposure to both active and passive smokers, an average smoking of twenty cigarettes a day results into intake of 2-4 μg of cadmium (WHO, 2011).

Municipal installations: Open burning, landfills or incineration of cadmium containing waste increases risk of cadmium in air particles (UNEP, 2015).

Drinking water: The contamination of drinking water due to the presence of cadmium in cadmium-containing solders, water coolers, taps, and water heaters (WHO, 2011).

• **Effects of Cadmium (Cd(II)) in the environment**

Effects on humans: Cadmium affects the human health due its high mobility and toxic nature. Liver dysfunction, disruption of hematopoietic and immune system, osteoporosis are reported as consequences of short-term exposure of cadmium. Other effects include the kidney dysfunction, DNA degradation, and cancer. Inhalation of relatively high concentration of cadmium in atmosphere causes great risk of lung cancer, other targeted sites for cadmium carcinogenesis are stomach, reproductive system, liver, and kidney (Waalkes, 2000; Lin et al., 2012; UNEP, 2013).

Effects on soil: The toxicity and bioavailability of cadmium in soil depends on the characteristics of soil. Cadmium enters the soil through composts, sludge or fertilizers (Kirkham, 2006). Cadmium enters the geochemical cycle through plants grown in cadmium loaded soil, consequently it enters into the food chain causing problem of bioaccumulation and biomagnification (Volesky, 2007; Gadd, 2009).

Permissible limits of Cadmium: World health organization (WHO) has recognized that the average weekly intake of cadmium present in food lies in the range of 0.7-2.8 μg/kg of body weight. According to EPA maximum contaminant level for cadmium exposure is 0.005 mg/L. WHO recommended in its guidelines that 0.003 mg/L of Cd is its threshold limit in drinking water.

2.8 Techniques Adopted for Removal of Heavy Metals

The various techniques adopted for removal of heavy metals are as follows:

• Chemical precipitation

The heavy metals from wastewater can be effectively removed by converting dissolved metal ions into insoluble solid phase with the precipitant. Heavy metals are typically precipitated in the form of hydroxides, sulphides or sometimes sulphates and carbonates. The resulting precipitate is then separated by sedimentation or filtration (Kurniawan et al., 2006; Chen et al., 2009; Fu and Wang, 2011). Lime $Ca(OH)_2$ is commonly used precipitant for removal of lead, metal precipitated is in the form of hydroxide. Recent studies on precipitation are limited and involve very complex procedures. The conceptual equation of chemical precipitation is as follows:

$$M^{2+} + 2\,(OH)^{-} \longleftrightarrow M(OH)_2 \downarrow \tag{2.2}$$

Where M^{2+} and OH^{-} represent the dissolved metal ions and hydroxide respectively, and $M(OH)_2$ represents the insoluble metal hydroxide.

• Ion exchange

This technique utilises the ion exchange resins (natural or synthetic) to exchange the cations with metal ions in wastewater. The reversible interchange of ions between the solid and liquid phases occurs in this process. Ion exchange can be applied for the recovery of valuable metals from the effluent. The uptake of metal ions is affected by the surrounding conditions of pH, temperature, initial metal concentration and contact time. By ion exchange either complete removal of ions or selective de-ionization can be performed. This method is generally being employed for recovery of trace amounts of metals from wastewater on a large scale. The sulphide cation exchanger exhibiting $SO^{-}{}_2H^{+}$ groups depict greater exchangeability and reversibility for the removal of Pb(II) from effluent solution. Also, the polystyrenesulphonic cation exchanger, chelating ion exchangers with phosphonic and sulphonic groups is widely used for Cd(II) ions removal (Dabrowski et al., 2004). As the functional groups of strongly acidic cationic resins consist of sulfonic acid, physiochemical interactions are assumed to occur during metal removal in the following equation:

$$\mathrm{nRSO_3H + M^+ \longleftrightarrow NRM^{n+} + nH^+} \tag{2.3}$$

Where (RSO_3) and M^+ represent the anionic group attached to the ion exchange resin and the cationic metal respectively, while n is the reaction coefficient depending on the oxidation state of metal ions. Despite its proper functioning, ion exchange process has limitation for requirement of the pre-treatment by removal of suspended solids from the secondary effluent. Additionally, particular ion-exchange resin is needed for the heavy metal ion which increases the operational cost of the process.

• **Coagulation-flocculation**

This process adopts the destabilization of suspended colloidal particles of the effluents into sediments by the coagulant. Coagulation is followed by flocculation of the unstable particles in form of larger agglomerates. Traditionally coagulation has been used to remove turbidity in water, but now it is used for removal of other contaminants including organic matter, metals etc. (Shammas, 2005). Generally ferric or alum salts are used as the coagulant for the proper cohesiveness of the suspended particles. Coagulation-flocculation process can treat metals from low to higher concentration such as below 100 mg/L and higher than 1000 mg/L. This process has high operational costs which limits it actual application.

• **Membrane filtration**

This process utilizes different types of membranes for the heavy metal removal from the solution. Depending on the size of particles membrane filtration can be classified as ultrafiltration, reverse osmosis, electrodialysis and nanofiltration. Membrane can be classified as per their material, pore size, and metal ions removal capacity. Ultratfiltration utilizes permeable membrane for separating heavy metals. Chitosan-enhanced membrane was used for removal of copper and zinc ions from the wastewater solution (Juang and Shiau, 2000). Micellar-enhanced ultrafilter was used for removing nickel ions from the synthetic solution (Kurniawan et al., 2006).

Reverse osmosis utilizes the osmotic pressure developed by the difference of solute concentration on the either sides of membrane, the reverse of normal osmosis process. The membranes used for reverse osmosis have a dense barrier layer in the polyamide material where most separation occurs. Na_2EDTA has been used for removal of copper and nickel ions from the wastewater solutions (Fu and Wang, 2011). The higher is the pressure

the higher is metal removal also it results into high energy consumption. Nanofiltration technology is the intermediate of ultrafiltration and reverse osmosis, it encapsulates the small pore bearing surface charged membranes such as polyvinyl alcohol (Ahn et al., 1999; Murthy and Chaudhari, 2008). Brackish water, sea water, and industrial effluents are mostly treated with the electrodialysis method. High suspended solids, metal ions make the membrane pores prone to blockage which causes irreversible membrane fouling. The membrane cannot be treated but can only be replaced by a new one, thus enhancing its operational cost (Tünay, 2004; Kurniawan et al., 2006).

• **Adsorption**

Adsorption can be defined as the process in which the molecules move from a bulk phase (solid, liquid or gas) onto the surface of another solid or liquid. Molecules that get adsorbed onto another surface are called as adsorbates, and the surface to which they are adsorbed is called as adsorbent. The adsorption process can occur at the interface of liquid-liquid, gas-liquid, gas-solid or liquid-solid surfaces. At the liquid-solid interfaces, the adsorption takes place into three types such as (a) physical adsorption, because of the vander-waals interaction, (b) chemical adsorption, because of formation of chemical bond between the adsorbate and the adsorption sites (c) exchange adsorption, because of the electrical attraction of adsorbate molecule onto the adsorbent (Kumar et al., 2008). The characteristics of an adsorbent are determined by the quantity of adsorbate it can accumulate, and also the rate at which the adsorption occurs, which is usually estimated by the adsorption isotherms. For an adsorbent to perform good, it is required to have a porous surface (resulting in high surface area) and less time should be taken in achieving an adsorption equilibrium. This makes it perfect for removing pollutants from the wastewater in considerable time (Kurniawan et al., 2006).

In order to understand the distribution of solute between the solid and liquid phase at equilibrium at constant fixed temperatures, mathematical models are useful, which are known as adsorption isotherms. The adsorptive metal uptake can be quantitatively evaluated by experimental equilibrium isotherms (Puranik and Paknikar, 1997; Namasivayam and Kavitha, 2002). The concentration of adsorbate in liquid phase and its concentration in solid phase are examined at constant temperature, to determine the equilibrium relationship. There are two widely accepted and linearized adsorption isotherm models used in the literature, namely Langmuir and Freundlich isotherms.

Langmuir isotherm model: Langmuir adsorption isotherm is based on assumptions such as: (a) uniform or monolayer coverage of the adsorbate on the adsorbent's surface. (b) all the adsorprion sites are identical (c) there are no lateral interactions between adsorbed

species (d) enthalpy of adsorption is independent of surface coverage. This provides an idea about the capacity of uptake, reflecting the equilibrium process behaviour (Langmuir, 1918; Duffus, 2002; Mohan et al., 2006; Febrianto et al., 2009).

The following equation explains the Langmuir isotherm model. Where Ce (mg/L) is the equilibrium concentration of metal in solution, q_e (mg/g) the amount of metal adsorbed on the adsorbent at equilibrium, q_{max} (mg/g), the monolayer capacity of biosorbent, K_L is the Langmuir constant, it relates to the capacity and energy of the adsorption.

$$q_e = \frac{q_{\max} K_L C_e}{1 + K_L C_e} \tag{2.4}$$

Another dimensionless parameter named separation factor (R_L) is introduced in Langmuir's model to indicate the favourability of the adsorption and is determined by the following equation

$$R_L = \frac{1}{(1 + bC_o)} \tag{2.5}$$

Where C_0 is the initial liquid phase concentration of adsorbate in equilibrium with the adsorbent and for favourable adsorption, and *b* is adsorption coefficient (Hall et al., 1966).

Freundlich isotherm model: Freundlich model has been developed for adsorption system with consideration of two factors, namely the lateral interaction between the adsorbed molecules and the heterogeneity of the energetic surface (Freundlich, 1906). The Freundlich model is based on the relationship between the metal uptake capacity q_e (mg/g) of adsorbent, and the residual (equilibrium) metal ion concentration C_e (mg/L). It assumes the sorbent to have a surface with a non-uniform distribution of sorption heat. The general form of the equation is as follows

$$q_e = K_F C_e^{1/n} \tag{2.6}$$

The linearized form of Freundlich isotherm model is given as:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \tag{2.7}$$

Where q_e (mg/g) is the concentration of metal ion on the biosorbent at equilibrium, C_e (mg/L) is the metal ion concentration in solution at equilibrium. K_F, the Freundlich constant and 1/n is the intensity of adsorption. The value of 'n' should be lying in the range of 1 to 10 for favourable adsorption. A smaller value of (1/n) indicates a stronger bond between adsorbate and adsorbent, while a higher value for K_F indicates higher rate

of adsorption.

• **Biosorption**

It is a physio-chemical metabolism-independent process of removal of substances (metals or metalloids) from the solution with the low cost biologically originating materials (Gadd, 2009). Biosorption is an emerging and promising technology for removal of heavy metal from contaminated wastewater. The researchers have been oriented towards using of inexpensive waste materials for the biosorption purpose such as: agricultural waste (sawdust, rice husk, peanut shells, tea waste, Coir fibers etc.) (Yang, 2005; Amarasinghe and Williams, 2007; Bulut and Tez, 2007; Conrad and Hansen, 2007; Naiya et al., 2009; Ronda et al., 2015), plant and animal derived biomass and algal biomass (sea weed, bael tree leaf, chitosan, crab shells etc.) (Niu and Volesky, 2003; Deng et al., 2007; Senthilkumar et al., 2007; Kumar and Gayathri, 2009; Nguyen and Juang, 2015; Kumar et al., 2016) microbial biomass (bacteria, fungi and yeast) (Puranik and Paknikar, 1997; Hawari and Mulligan, 2006; Tunali et al., 2006; Zhou et al., 2007; Wei et al., 2009; Guo et al., 2010; Li et al., 2010; Çolak et al., 2011; Oves et al., 2013; Hlihor et al., 2016). The major advantage is its high effectiveness in metal ion removal and usage of inexpensive biosorbents. The high costs of the traditional physio-chemical methods of heavy metal removal limit their application. Biosorption has therefore promoted as potential biotechnological approach of treating heavy metal loaded wastewaters and industrial effluents.

Factors affecting Biosorption: Similar to the adsorption process, biosorption also depends upon the surrounding experimental conditions (Veglio and Beolchini, 1997; Gadd, 2009; Park et al., 2010) such as:

pH: the solution pH regulates the availability of ions in the solution and also the charge of the functional groups on the adsorption sites, deciding the competition between the ions for getting adsorbed onto the surface of adsorbent (Al-Garni, 2005; Gong et al., 2005).

Temperature: the surface activity, affinity and kinetic energy of the adsorbate molecules increases with the increasing temperature, but upto an optimum level, otherwise it can destroy the physical structure of the adsorption sites (Marqués et al., 1991; Al-Asheh and Duvnjak, 1995; Zhang et al., 2013).

Contact time and agitation speed: increasing agitation speed enhances the rate of contact of the metal ions with the adsorbent surface, but too much agitation can lead to distortion of the adsorption sites (Park et al., 2010; Çolak et al., 2011).

Initial metal concentration: Enhances the quantity of metal ions adsorbed onto the surface of adsorbent initially with the increase in metal concentration but decreases thereafter due to increase in electrostatic interaction, involving sites of low affinity for metal ions (Puranik

and Paknikar, 1999; Fomina and Gadd, 2014).

Biosorbent nature and type: type and availability of binding sites, biomass dosage, structure, size and composition (Park et al., 2010; Fomina and Gadd, 2014).

Table 2.2 depicts the list of microbes with their metal removal efficiency and their optimum conditions of biosorption.

2.9 Future Perspectives

Biosorption describes the microbial ability to interact and accumulate the heavy metals from its surrounding areas. This gives scope of an extensive research in the field of micro-bioremediation. New biosorbents such as novel microorganisms, algae, agricultural material, industrial waste, plant material, and natural residues can be investigated for their biosorption potential. In terms of the scaling up of biosorption process, biosorbents are needed to be modified and manipulated for the improvement of biosorption technique. Pretreatment of biomass with acid, alkali, organic solvents of alcohol, acetone, toluene, and inorganic salts of NaCl and Na_2CO_3 heating, autoclave, and lyophilization can be done to detect and improve the performance of biomass (Nagase et al., 2005; Yazıcı et al., 2008; Yang and Chen, 2008; Ngah and Hanafiah, 2008).. Immobilisation of biomass with activated carbon, beads, alginate, polyacrylamide, formaldehyde etc. can be helpful in upscaling of the biosorption study. Magnetic modification, as well as clay and bacterial biofilms modification of bacterial biomass and bacterial amyloids, can improve the biosorption capacity of the existing biosorbents. Although, with pretreatment biomass loss can occur and limit the enhancement of biosorption capacity, polyethylenimine (PEI) and acrylic acid modified fungal biomass of *Penicillium chrysogenum* depicted significant increase in cell functionalization and sorption capacity for Cu, Pb, and Ni (Zouboulis et al., 2003; Deng and Ting, 2005).

Process parameters influencing the performance of biosorption (in batch and column mode operation) anticipate the biosorption capacity of a biosorbent. The information about the biosorption capacity obtained from a batch system gives the fundamental details about the effectiveness of a biosorbent. Microbial capability of removing heavy metals in continuous column mode can give a better scenario of predicting their application on upscale for the wastewater treatment industries (Kumar and Chakraborty, 2009; Colak et al., 2011; Hlihor et al., 2016). Limited literature is available on the biosorption study of different biosorbents in the native and modified state with batch and continuous column mode operation. Therefore, an immense scope exists for investigating the biosorption potential of different biosorbents in the removal of various heavy metals. Also, modification of bacteria at the molecular level can improve their nature of interaction with the heavy metals. Recent

development in genetic engineering techniques have modified the functionality of microbes by regulating the metal-binding proteins, metalloregulatory proteins, phytochelatins, metallothioneins and other short peptides have opened up new avenues for micro-bioremediation (Yang et al., 2015).

2.10 Concluding Remarks

Microorganisms are a feasible solution for removal of heavy metals as compared to the conventional physiochemical methods because they perform different bioaccumulation and biosorptive processes. The metabolic functions enable the microorganisms to either incorporate the metal ions inside the cellular surface or accumulate on the microbial cell surface. They have great potential to remove metal in a better way than the exixting conventional technologies. Different wastes such as industrial waste, wastewater, and soils are loaded with heavy metals, additionally these wastes are also rich in microorganisms. Consequently, the wastes serve as a rich source of robust microbes resistant to heavy metals. Furthermore, the microbes isolated can be used for micro-bioremediation and commercializing on larger scale for the metal removal processes. The application of microbiological and biotechnological studies in the field of wastewater management, heavy metal removal and sustainable waste management will be justified. Thus, adding a new dimension to the effective interdisciplinary research. This can prove to be an efficient solution for the heavy metals removal in the most effective and economical way.

The current technologies for waste management and wastewater treatment are sustainable solution of waste management and bioremediation. They are aiming towards the utilization of microbes in micro-bioremediation with biosorption process. Successful studies have been reported focusing the isolation and characterization of microbes from various waste sources such as wastewater, soil, sewage sludge and industrial wastes. Therefore, in current study rotary drum compost of water hyacinth was used as a source of isolation of microbes. The application of isolated microbial strain was investigated in the domain of heavy metal removal by biosorption process. The bacterial strain was utilised as a biosorbent in its native or non-pretreated state as well after pre-treatment. Biosorption of lead and cadmium was investigated for the metal removal capacity by the biomass of the newly isolated bacterial strain. The batch and continuous column mode operation was performed for the biosorption study. The process parameters influencing the biosorption were evaluated in anticipation of the practical application of biomass as an inexpensive, easily cultivable and efficient biosorbent in upscale for bioremediation technology in future.

"Environmental pollution is an incurable disease. It can only be prevented."

Barry Commoner

3

Materials and Methods

Different experimental approaches were used to accomplish the stipulated objectives. The research was carried out in different phases. The detailed methodology is mentioned in this chapter.

3.1 Experimental Design

In order to accomplish the objectives, the proposed research work was carried out in four different phases (Figure 3.1). In phase I, experiments were conducted on the rotary drum composting of water hyacinth in different ratios. Compost analysis was performed to determine the best combination of waste materials. In phase II, microbiological studies of the best trial of water hyacinth compost was performed, microbial diversity of the best combination was performed also, the isolation and identification of the bacteria was performed from the best trial with the 16S rRNA sequencing method. In phase III, the application of isolated bacteria was investigated by the biosorption studies of Pb(II) and Cd(II) in batch system. The live (non-pretreated) and dried (pretreated) form of bacterial biomass was investigated for biosorption of Pb(II) in batch system, whereas, dried form of bacterial biomass was investigated for biosorption of Cd(II) in batch system. In phase IV, the continuous or column performance of bacterial biomass was detected for biosorption of Pb(II).

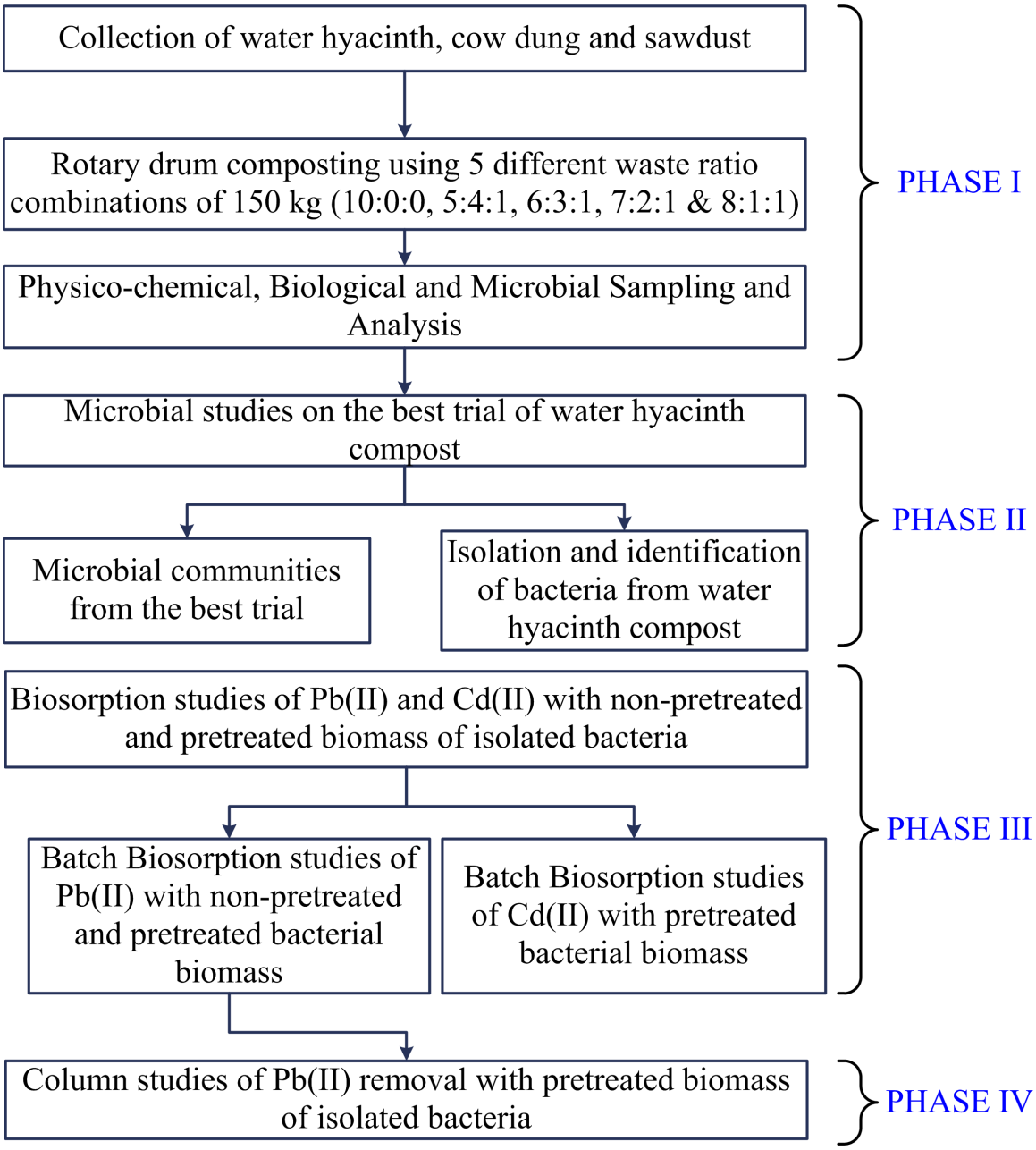


Figure 3.1: Experimental design of research work

3.2 PHASE I: Microbial Population, Stability and Maturity Analysis of Rotary Drum Composting of Water Hyacinth

3.2.1 The Compost Material

Different waste mixtures were prepared by mixing of water hyacinth, cow dung and sawdust. Water hyacinth was collected from Amingaon industrial area near Indian Institute of Technology Guwahati (IITG), Assam, India (Figure. 3.2 (a)), Cow dung was obtained from dairy farm near to the IITG campus, Sawdust was purchased from nearby saw mill. Sawdust and cow dung were mixed thoroughly to form fine powder. Water hyacinth was shredded into small pieces of order 1 cm so as to maintain proper aeration and control the moisture. Sawdust and cow dung powder and water hyacinth were mixed thoroughly in five different proportions as detailed in Table 3.1.

Figure 3.2: (a) Collection site near Amingaon Industrial Area, Guwahati, India (b) Feedstock materials

3.2.2 Feedstock Preparation

Different waste combinations of the organic waste such as water hyacinth, cow dung and saw dust were mixed together in the ratio of 10:0:0, 5:4:1, 6:3:1, 7:2:1 and 8:1:1 making up the total volume of 150 kg for rotary drum composting as shown in Table 3.1.

The cow-dung brought from the nearby dairy farms was mixed with sawdust by hand to ensure no big clumps were formed. Water hyacinth was shredded into 1-2 cm size using electric shredder and manual chopping. This was done to ensure proper degradation along with aeration. Then all three were mixed together and feed to the drum composter as shown in (Figure. 3.2 (b)). The study of the microbial activity and the physiochemical parameters were done experimentally.

Table 3.1: Waste composition of different mixtures

Trial	Composition/ Ratios	Water hyacinth (kg)	Cow dung (kg)	Saw dust (kg)
Trial 1	08:01:01	120	15	15
Trial 2	07:02:01	105	30	15
Trial 3	06:03:01	90	45	15
Trial 4	05:04:01	75	60	15
Trial 5	10:0:0 (Control)	150	0	0

3.2.3 Rotary Drum Composting

Water hyacinth collected from Amingaon industrial area was chopped into 1 cm to 2 cm size and mixed along with cow dung and sawdust. Figure 3.3 depicts the pilot-scale rotary drum composter of 550 L capacity being operated on batch mode. The capacity of the pilot scale rotary drum composter was decided keeping in view of the capability of a single person who can handle around 150 kg of waste by manual rotation. The main unit of the composter is fabricated with 4 mm thick metal sheet with 1.22 m (length) and 0.76 m (diameter). An anti-corrosive coating is present on the inner side of drum. The drum is mounted on a metal stand with rubber rollers. In order to maintain proper mixing, agitation and aeration of the wastes during rotation, 40 mm $\times$ 40 mm angles are welded longitudinally inside the drum. Two adjacent holes of 10 cm each are present on top of the drum to remove excess water (Kalamdhad et al., 2008)

Manual turning was done after every 24 h through one complete rotation of the rotary drum to ensure that the material on the top portion moved to the central portion, where it was subjected to higher temperature (Kalamdhad and Kazmi, 2009). After that, aerobic condition was maintained by opening the top half side doors of the two circular faces.

3.2.4 Sampling and Analysis

A total of 1 kg sample was collected for complete analysis. Grab sampling was carried out for sampling. This sample was prepared by taking representative samples from 9 different points mainly from the middle portion and end terminals of the pilot-scale rotary drum composter after drum rotation so as to ensure homogenized sample. These homogenized samples were collected on 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days of the composting process. The 1 kg sample was then divided into two parts viz. (A) for physio-chemical analysis (800-900 g) and (B) for biological and microbial analysis (150-200 g). The sub-samples (A) were air dried immediately, ground to pass through 0.2 mm sieve and stored for physio-chemical analysis. The sub-samples (B) were either used or stored at 4°C for

biological analysis of the wet sample within 2 days.

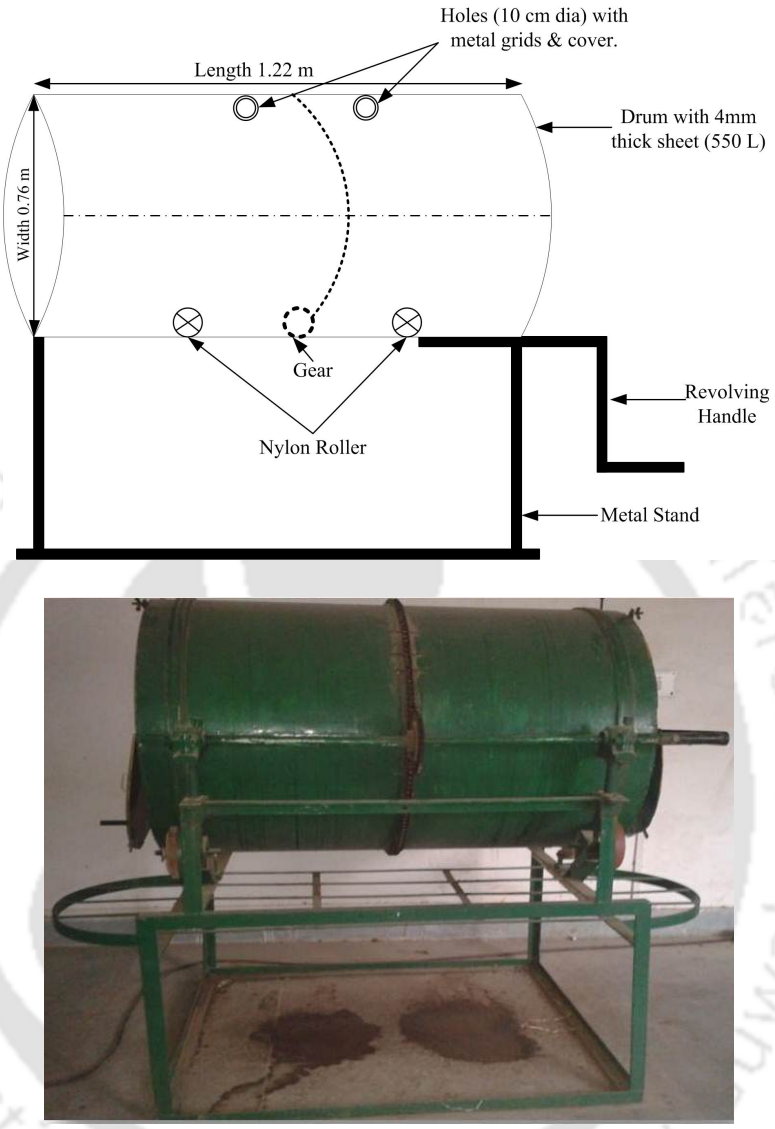


Figure 3.3: Pilot Scale Rotary Drum Composter (a) Schematic design (b) Pictorial view

3.2.4.1 Physico-chemical Analysis

Different experimental methods were used in the study to accomplish the stipulated objectives. physio-chemical analysis of the solid waste samples including water hyacinth, cow dung and sawdust were carried out in Centre for Environment Laboratory, IIT Guwahati. Experimental procedures of physio-chemical parameters are explained below.

1. **Moisture content (IS:10158-1982)**

   - Take initial weight of the plate as W_1 g.
   - Weigh 200 $\pm$ 0.1 g of fresh sample in the plate and kept it in an oven operating at a temperature of 105°C.
   - After 24 h the plate was taken out and the final weight of plate with sample was taken as W_2 g.
   - Then the moisture content (MC) of the sample was calculated as:

$$\text{MC}(\%) = \left(\frac{200 - (W_2 - W_1) \times 100}{200}\right) \tag{3.1}$$

2. **pH (IS:10158-1982)**

   - About (10 $\pm$ 0.1g) of ground sample (screened through 0.22 mm sieve) was taken and dissolved into 100 mL of distilled water.
   - Kept it in a horizontal shaker for 2 h.
   - Then kept it for about $1/2$ h for settling down the solids.
   - Standardize the pH meter (ambient temperature) by immersing the electrode in buffer solution of known pH, normally 4.0 and 7.0.
   - Read the pH and adjust correctly with the knob, till the reading indicated the correct value for pH of buffer solution.
   - Rinse the electrode with distilled water.
   - Then the pH of the suspension was measured with pH meter.

3. **Electrical conductivity (EC) (IS:10158-1982)**

   - About (10 $\pm$ 0.1g) of ground sample (screened through 0.22 mm sieve) was taken and dissolved into 100 mL of distilled water.
   - Kept it in a horizontal shaker for 2 h.
   - Then the diluted sample was filtered through Whatman 42 no. filter paper.
   - Rinsed the electrode with distilled water.
   - The electrical conductivity, EC of the filtered sample was measured with conductivity meter, and expressed as dS/m.

4. **Volatile solids (IS: 10158-1982)**

   - Initial weight of the crucible was taken as W_1 g.
   - Weigh (10 $\pm$ 0.1g) of ground sample (screened through 0.22 mm sieve) in crucible and kept it in a muffle furnace operating at a temperature of 550-600°C for 2 h.
   - After 2 h crucible was taken out of the muffle furnace and kept in desiccators for 1/2 h for cooling and then final weight of crucible with sample was taken as W_2 g.
   - Then the Volatile Solids (VS) content of the sample was calculated as:

$$VS(\%) = \frac{(10 - (W_2 - W_1)) \times 100}{10} \tag{3.2}$$

3.2.4.2 Biological Analysis

1. **Soluble Biochemical Oxygen Demand (APHA, 2005)**

   - About 10 $\pm$ 0.1g of fresh compost was taken in a conical flask and dissolved in 100 mL of distilled water.
   - The flask was kept in a horizontal shaker for 2 h
   - Then it was filtered using whattman 42 no. filter paper.
   - The supernatant of samples were taken and analysed for BOD test using BOD_5 test calculation:

$$BOD_5\,(\text{mg/L}) = \frac{(D_1 - D_2)}{P} \times 1000 \tag{3.3}$$

   Where D1= Initial DO of sample in mg/L D2= Final DO of Sample after 3 days incubation in mg/L P = Sample volume (mL) diluted to 300 mL with dilution water

2. **Chemical Oxygen Demand (APHA, 2005)**

   - About 10 $\pm$ 0.1 g of fresh compost was collected in a conical flask and dissolved in 100 mL of distilled water.
   - The flask was kept in a horizontal shaker for 2 h.
   - Then it was filtered using Whatman 42 no. filter paper.

- The supernatant of samples were taken and analysed for COD using closed reflux method.
- 1.5 mL of $K_2Cr_2O_7$ + 2.5 mL of sulphuric acid reagent were added to COD vial.
- It was digested for 2 h in COD digestor at 150°C and cooled down to room temperature
- Then cooled and titrated against ferrous ammonium sulphate (FAS) using ferroin indicator till colour changes from green to wine red.

3. **CO_2 evolution by Soda-Lime method (Kalamdhad et al., 2008)**

- About (25 $\pm$ 0.1g) of fresh compost sample was taken in 1 litre PVC airtight container.
- About 10 g of Oven dried (105°C) soda lime (1.5-2.0 mesh) soda-lime was taken in a 100 mL beaker and it was placed in the above container. The initial weight of the soda-lime taken as (W_1) g.
- The container with soda-lime beaker was kept in an incubator, set at temperature of 25°C.
- After 20-24 h the soda-lime was taken out and oven dried it again, then the final weight is noted down as (W_2) g.
- The difference in mass of soda lime will give the amount of CO_2 absorbed. Calculation:

$$\text{CO}_2 \text{ evolution rate } (\text{mg/g VS/day}) = \frac{(W_2 - W_1)}{W \times T} \times 1000 \qquad (3.4)$$

Where W_1 = Initial weight of the soda-lime (g), W_2 = Final weight of the soda-lime (g), W = Weight of compost sample taken (g), T = Time duration of incubation (h)

4. **Oxygen uptake rate (OUR) (Kalamdhad et al., 2008)**

The oxygen uptake rate (OUR) was performed according to the method described by (Lasaridi and Stentiford, 1998). The OUR was measured in a liquid suspension of compost (5-8 g of compost in 500 mL of distilled water added with $CaCl_2$, $MgSO_4$, $FeCl_3$ and phosphate buffer at pH 7.2) the solution was kept in suspension by placing it on the magnetic stirrer at constant temperature by keeping the whole assembly into the water bath held at 30°C . During this time, the dissolved O_2 of the suspension was

continuously observed through the digital DO meter attached to it. The oxygen consumption rate was calculated by taking the difference of DO with respect to the time intervals and this value was quoted as the OUR in mg O_2/g VS/h.

3.2.4.3 Heavy metals analysis

All the heavy metal analysis were accomplished using atomic absorption spectrophotometer AAS (Varian Spectra 55B). All the experiments were done in triplicates, and the result is depicted as the means of them. Total metal was analysed by digesting 0.2 g of dried sample in 10 mL of H_2SO_4 and $HClO_4$ (5:1) mixture in a block digestion system for 2 h at 300°C (Pelican equipment Chennai-India).

For water-soluble heavy metal analysis, 2.5 g of sample was extracted with 50 mL of distilled water (sample: solution ratio = 1:20) and kept in a shaker at 100 rpm for 2 h at room temperature. DTPA (Diethylenetriamine penta acetic acid), 4 g of ground sample (sieved through 0.22 mm sieve) was mixed with 40 mL of 0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M (Triethanolamine) (pH 7.3) and mechanically shaken at 100 rpm.

The leachability of heavy metals was determined by TCLP (Toxicity characteristic leaching procedure), following the EPA method 1311, 5 g of solid sample (size less than 9.5 mm) was mixed with 100 mL of acetic acid at pH 4.93 $\pm$ 0.05. The sample and solution were taken in a ratio of 1:20 in 125 mL of reagent bottle and kept at 30 $\pm$ 2 rpm for 18 h at room temperature. The remaining suspensions were filtered by Whatman no. 42 filter paper after being centrifuged at 10,000 rpm for 5 min. The samples were stored in a specimen tube at 4°C for analysis of selected heavy metals.

The calculation of extraction efficiency of DTPA-extractable heavy metals, the following equation was used (Singh and Kalamdhad, 2013a).

$$\text{Extraction effeciency } (\%) = \frac{C_{\text{DTPA}}}{C_{\text{Total}}} \times 100 \tag{3.5}$$

where C_{DTPA} depicts the concentration of DTPA extractable heavy metal,C_{Total} signifies the concentration of total heavy metals.

3.2.4.4 Microbial Analysis

1. **Sampling for microbial analysis** Samples were collected from mid area, and end terminals from every fourth day such as 0, 4, 8, 12, 16, 20th day after turning the rotary drum composter. The sample for microbial count analysis was prepared by mixing 10 g compost or waste material with 90 mL of sterile distilled water containing

0.85% (w/v) sterile sodium chloride in 250 mL of Erlenmeyer flasks, the solution is mixed mechanically at 150 rpm for 2 h at 25°C. Finally, the waste suspensions were diluted serially and used for microbial counts on appropriate media.

2. **Culture media and conditions**

- For bacterial growth nutrient agar medium was used, total count of the prokaryotes was examined. Petri plates were poured with the nutrient agar medium and kept in the BOD incubator for 24-48 h at 25°C for mesophilic bacteria and for 24 h at 55°C for the spores of bacteria. Cycloheximide (0.2 g/L) was added in order to restrict the growth of fungi.
- For actinomycete growth, actinomycete isolation agar was supplemented with 2 g sodium caesinate, 0.1 g L-Asparagine, 4 g sodium propionate, 0.5 g K_2PO_4, 0.1 $MgSO_4$, 0.001 g $FeSO_4$ and 5 mL glycerol per litre and cycloheximide (0.2 g /L) (for fungal growth inhibition). Final petri plates poured with this medium were incubated in the BOD incubator at 25°C for 4-5 days.
- Fungal count was done in the Sabouraud 4% dextrose agar with 5 g peptone from casein, 5 g peptone from meat, 40 g D (+) Glucose per litre. Prepared plates were incubated at 25°C for 3-4 days.
- Streptomycetes were counted in the ISP medium No.4 supplemented with 10 g starch soluble, 1 g K_2HPO_4, 1 g $MgSO_4 \cdot 7\,H_2O$, 1 g NaCl, 2 g $(NH_4)_2SO_4$, 2 g $CaCO_3$ per litre and 0.2 g/ L of cycloheximide for fungal inhibition.
- Lauryl tryptose broth and EC medium respectively were used for total coliforms (TC) and fecal coliforms (FC) analyses in the culture tube by using the most probable number (MPN) method (Federation and Association, 2005).

3.3 PHASE II: Microbial Studies on the Best Trial of Water Hyacinth Compost

The microbial analysis of water hyacinth compost was first performed by the enumeration and identification of microbial communities followed by the isolation and identification of bacteria by 16S rDNA analysis. First step towards pure culture is by selecting appropriate growth media. The following media given in (Table 3.2) were selected for obtaining efficient media by comparing the ability of the media to support growth of maximum number of bacterial species and consistency of performance in supporting growth of target microbes.

3.3.1 Microbial Succession (DNA) From Best Trial

DNA was isolated using modified Xcelgen Soil gDNA kit. Quality of gDNA was checked on 1% agarose gel (loaded 5 μL) for single intact band. The gel was run at 110 V for 30 mins. 1 μL of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio. The DNA was quantified using Qubit dsDNA BR Assay kit (Thermo Fisher Scientific Inc.). 1 μL of each sample was used for determining concentration using Qubit® 2.0 Fluorometer.

3.3.2 Culture Dependent Bacterial Identification

For culture-dependent bacterial identification samples were collected from mid area, and end terminals from every fourth day such as 0, 4, 8, 12, 16, 20^{th} day after manual turning. The microbial count was done by adding 10 g of waste or compost into 90 mL of sterile distilled water containing 0.85% (w/v) sodium chloride solution in 250 mL Erlenmeyer flasks. The solution was mixed mechanically at 150 rpm for 2 h at 25°C. Finally, the waste suspensions were serially diluted and used for microbial counts on all four media separately. Spread plate technique was performed to enumerate the bacterial colonies in the compost sample (Figure 3.4). Plates were then incubated in an inverted position for 24-72 h at 25°C. Plates with a lower number of distinguished bacterial colonies were picked up for purification and separation by quadrant streaking. Initially, streaking was done on every fourth day, and then the time interval was increased to 15 days. Plating was done till the final 20th day of composting with the observance of more number and sustainable bacterial colonies in different media. Serially diluted samples were plated on Mac Conkey Agar for counting *Escherichia coli* number in plates kept for 24-48 h at 37°C. Glucose Minimal media depicted the maximum growth of bacterial colonies giving twelve distinguished robust bacterial isolates. Morphological testing of these twelve isolates was performed with Hi-media bacterial kit for the biochemical tests. The identifications were confirmed using Bergey's Manual of systematic bacteriology (Aneja, 1999).

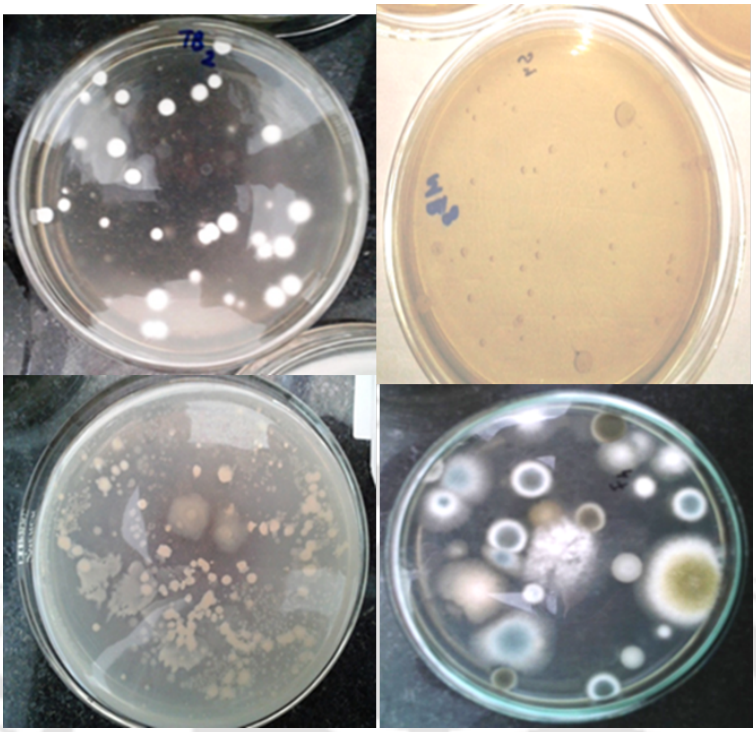


Figure 3.4: Microbes in Petri-plates

3.3.2.1 Media Optimization

The first step towards the isolation of bacterial pure culture was by selecting appropriate growth media. The medium that is used for bacterial growth can have a major impact on the isolation efficiency. As the source of isolation was water hyacinth compost, appropriate growth medium for bacteria was unknown. As mentioned in Table 3.2, four different types of media including two growth media and two minimal media were tested for maximum number of bacterial species and consistency of performance in supporting growth of targeted microbes. Cycloheximide (0.2 g/L) was added as antibiotic in all media to avoid contamination by fungi.

Different types of media are mentioned in the literature, on the basis of the goal of isolation and its source. Growth can be affected by components of media, difference can be observed in same sample cultured in nutrient enriched and minimal media. Selection of suitable growth medium is crucial step for the isolation of novel strains from a waste source, which is enriched with heterotrophic unidentified bacteria. However, a comparative study of bacterial growth and isolation in different nutrient media is missing in literature.

Table 3.2: Media compositions and their literature references

Media name	Ingredients	Reference
Nutrient agar	Peptone (5 g/L) , meat extract (1 g/L), Yeast extract (2g/L), NaCl (5g/L), agar (20 g/L)	(del Carmen Vargas-García et al., 2012)
Typtone Soy agar (Himedia)	Casein (17 g/L), papaic digest of soyabean meal (3g/L), NaCl (5 g/L), dextrose (2.5/L), K_2HPO_4 (2.5 g/L), agar (20 g/L)	(Parungao et al., 2007)
Sucrose minimal low phosphate media	Sucrose (1%),$(NH_4)_2SO_4$ (0.1%), K_2HPO_4 (0.05%),$MgSO_4$ (0.05%), NaCl (0.01%), Yeast extract (0.05%), $CaCO_3$ (0.05%), agar (20 g/L)	(Sheng et al., 2008)
Glucose minimal media	Glucose (1%), $(NH_4)_2SO_4$ (0.1%), K_2HPO_4 (0.05%), $MgSO_4$ (0.05%), NaCl (0.01%), Yeast extract (0.05 %), $CaCO_3$ (0.05%), agar (20 g/L)	*This media was investigated during the study by changing sugar of Sucrose minimal low phosphate media to glucose

3.3.2.2 Biochemical Tests

1. **Gram Staining:** For gram staining Himedia K004-1KT capsules satin kit was used. S025 Nigrosin stains 10% w/v 100 mL. S021 Methylene blue 125 mL. A loopful of culture was placed on glass slide and stained with 10% w/v Nigrosin stain (S025). The smear was mixed well and allowed to air dry and then fixed with gentle heat. Thereafter, the smear was stained with the methylene blue (S021) for 30 s. Rinsed rapidly with water and air dried. The glass slide was observed under the immersion oil lens. Gram positive bacteria: Stained dark purple colour due to retaining the primary dye called crystal Violet in the cell wall. Gram negative bacteria: Stained pink due to retaining the counter staining dye called safranin.

2. **Capsule stain:** For capsule detection, capsule stains kit (K004) was used. As smear of microbial culture was prepared on a glass slide. Drying and heat fixation was done with gentle heating. The slide with bacterial film on upper side was kept over a beaker with boiling water. When large droplets condensed on the lower side of the slide the smear was flooded with Schaeffer and Fulton spore stain A (S028). Finally the slide

was steamed for 3-6 min and rinsed under running tap water. Counter staining with Schaeffer and Fulton spore stain B (S029) was done for 30 s. The slide was washed with water, dried and observed under immersion oil lens.

3. **Spore staining:** Schaeffer and Fulton's spore stain kit K006-1Kt (Himedia) was used for detection of spores in the microbial culture. Following steps were followed for the process: Smear microbial culture on a glass slide and heat fix, cover it with a square of blotting paper. Saturate the blotting paper with carbolfuchsin and steam for 5-10 min, keep the paper moist and add 2 drops dye as required. Alternatively, the slides may be steamed over a container of boiling water. Remove the blotting paper and decolorize the film with acid-alcohol for 1 min, rinse with tap water and blot dry. Dry a thin even film of saturated aqueous nigrosin on the slide. Examine the slide under the oil immersion lens (1,000 $\times$) for the presence of endospores. Vegetative cells are colourless, endospores are red, and the background is black.

4. **Catalase test:** The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. ($2\,H_2O_2 + \text{Catalase} \longrightarrow 2\,H_2O + O_2$). This reaction is evident by the rapid formation of bubbles. There are many applications and method variations of the catalase test. These include the slide or drop catalase test, the tube method, the semi quantitative catalase for the identification of *Mycobacterium tuberculosis*, the heat-stable catalase used for the differentiation of *Mycobacterium* species, and the capillary tube and cover slip method. The following steps were followed for the test. Place a microscope slide inside a petri dish. Keep the petri dish cover available. Use a sterile inoculating loop collected a small amount of organism from a well-isolated 18- to 24 h colony and placed it onto the microscope slide. This is particularly important if the colony isolate was grown on agar containing red blood cells. Carryover of red blood cells into the test may result in a false-positive reaction. Use a dropper placed 1 drop of 3% H_2O_2 onto the organism on the microscope slide.. Immediately cover the petri dish with a lid to limit aerosols and observe for immediate bubble formation (O_2 + water = bubbles). Observe the formation of bubbles against a dark background to enhance readability. Positive reactions are evident by immediate effervescence (bubble formation). Place microscope slide over a dark background and use a magnifying glass of microscope and observe weak positive reactions. Use a microscope, placed a cover slip over the slide and view under 40 $\times$ magnifications.

5. **Oxidase test:** Oxidase test using N, N,N,N-tetramethyl-p-phenylenediamine reagent (Himedia GRM445), Kovács oxidase reagent (1%), tetra-methyl-p-phenylenediamine

dihydrochloride, in water was performed. The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colourless reagent becomes an oxidized coloured product.

6. **Filter spot method:** Following steps were followed for the test: Pick a microbial colony (18 to 24 h culture) by a loop and rub onto a small piece of filter paper. Place 2 drops of 1% Kovács oxidase reagent on the microorganism smear. Observe colour changes. Bacteria show dark purple within 5 to 10 s oxidase positive. Microorganisms are delayed oxidase positive when the colour changes to purple within 60 to 90 s. Microorganisms are oxidase negative if the colour does not change or it takes longer than 2 min. Motility test For motility test Mannitol Motility Test medium (Himedia M770) was used: Use a sterile needle to pick a well-isolated colony and stab the medium to within 1 cm of the bottom of the tube. Be sure to keep the needle in the same line as it entered as it is removed from the medium. Incubate at 35°C for 18 h or until growth is evident. A positive motility test is indicated by a diffuse cloud of growth away from the line of inoculation.

7. **Voges-Proskauer Test:** Barrett's reagent A: 5% (w/v) a-naphthol in absolute ethanol. Barrett's reagent B: 40% (w/v) KOH in deionized water (this might be replaced by a 40% (w/v) NaOH solution) Reagents must be prepared fresh. Reagents are also referred to as VP-1 and VP-2 or VP-A and VP-B. Sterile culture tubes containing 5 mL of MR-VP broth. Sterile culture test tubes and control bacterial strains (commonly used controls are *Escherichia coli* and *Enterobacter aerogenes*) from trypticase soy agar or broth inoculation loop (disposable or have additional equipment available for sterilization of the inoculation loop) and transfer pipettes. Following method was followed for the test: Add 0.6 mL of Barrett's reagent A and 0.2 mL of Barrett's reagent B carefully in a test tube. Shake the tube for 30 s to 1 min, expose the medium to atmospheric oxygen for the oxidation of acetoin to obtain a colour reaction. Allow the tube to stand for at least 30 min. Within 1 h, compare the test result to control cultures to determined culture is VP positive or VP negative. Delayed reading of the result may lead to an erroneous reading, over time a-naphthol and KOH may react to give a copper-like colour. Bacteria fermenting sugars via the butanediol pathway produce acetoin (i.e., acetyl methyl carbinol or 3-hydroxybutanone) as an intermediate which can be further reduced to 2,3 butanediol. In the presence of KOH the intermediate acetoin is oxidized to diacetyl, a reaction which is catalyzed by a-naphthol (2). Diacetyl reacts with the guanidine group associated with molecules contributed by peptone in the medium to form a pinkish-red-coloured product. The a-naphthol in

Figure 3.5: Bacterial identification tests in laminar air flow

the Barritt's modification of the VP test serves as a colour intensifier.

8. **Methyl red solution:** Methyl red and Voges-Proskauer test using MR-VP Medium (Himedia M070). Following steps were followed: Completely dissolved 0.1 g of methyl red in 300 mL of ethanol (95%). Add 200 mL of deionized water to make 500 mL of a 0.05% (w/v) solution in 60% (v/v) ethanol. Store the prepared methyl red solution at 4°C. Transfer 2.5 mL of culture into a new sterile culture tube. Add 5 drops of the methyl red reagent. Compare the test organism to the control cultures to immediately interpret the result. MR positive, false-negative results or an inconclusive orange colour may occur due to insufficient length of incubation. In this case, it is recommended that the test can be repeated with a culture that was incubated for an additional 24 to 48 h. An increase in glucose concentration in the medium might lead to false-positive results in the MR test. Clark and Lubes showed that increased glucose concentration also led to high hydrogen ion concentration in high-ratio cultures.

9. **Oxidative-fermentative test used media with glucose:** Oxidation-fermentation reaction test using basal medium (Himedia M395), Peptone (tryptone) 2.0 g, Sodiumchloride 5.0 g, Glucose (or other carbohydrate) 10.0 g Bromthymol 0.03 g, Agar 3 g, Dipotassium phosphate 0.30 g. The oxidative-fermentative test is used to determine gram-negative bacteria that metabolize carbohydrates oxidatively by fermentation, or are nonsacchrolytic and therefore have no ability to use the carbohydrate in the media. After the medium is autoclaved at 121°C for 15 min, a filter sterilized solution of 10% solution of carbohydrate is aseptically added to the medium to a final concentra-

tion of 1%. The sterile medium containing the carbohydrate is aliquoted aseptically into sterile test tubes and cooled unslanted as stabs. Some procedures call for the addition of 10 g/L of carbohydrate to the medium prior to sterilization. The medium is then dissolved by heating to a boil on a hot plate or by steaming for 20 min prior to aliquoting into test tubes. The medium in test tube is then steamed for 20 min in place of autoclaving to prevent break down of the carbohydrate. Basal medium is commercially available in a premixed form from biological supply companies. The carbohydrate source is not included and must be added as stated above.

3.3.3 Culture Independent Bacterial Identification

Isolation and identification of bacterial strain was performed by extraction of genomic DNA, PCR amplification of the isolated DNA and DNA sequencing to compare the nucleotide sequence with the Genbank database. Following steps give the detailed explanation of the process.

(a) Genomic DNA isolation from bacterial isolates: Genomic DNA was extracted from anexic culture pellets using GeNei DNA Purification kit as per manufacturer's instruction. The purity of DNA was then checked by horizontal gel electrophoresis in 0.8% agarose gel (Figure 3.6). (b) PCR amplification and 16S rDNA analysis: For PCR amplifications 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (forward) and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (reverse) primers were used. Reactions were performed in 50 μL volumes using Ready mix Taq PCR reaction Mix (Sigma Aldrich Ready Mix Taq PCR mixture) in PCR-peQLab thermocycler and following a protocol with a denaturation step at 94°C for 5 min, followed by 35 amplification cycles (95°C, 60 s; 52°C, 60 s; 68°C, 90 s) and a final extension step at 68°C

Table 3.3: Composition of PCR Reaction Mixture

Reagent	**Volume**	**Final Concentration**
2× Ready Mix Taq PCR Reagent Mix	25μL	1.5 units Taq DNA polymerase, 10 mM Tris-HCL, 50 mM KCL, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, stabilizers
Forward primer	1 μL	0.1-1.0 μM (15-30 bases in length)
Reverse primer	1 μL	0.1-1.0 μM (15-30 bases in length)
Template DNA	3 μL	–
Water	20 μL	–
Total Volume	50 μL	

for 10 min. The amplification products were cleaned using the Gen-Elute PCR Clean-Up kit (Sigma-Aldrich, St. Louis, MO, USA), sequenced and compared to the nucleotide database at GenBank by using BLAST (NCBI). (Table 3.3) depicts the reaction mixture during PCR reactions.

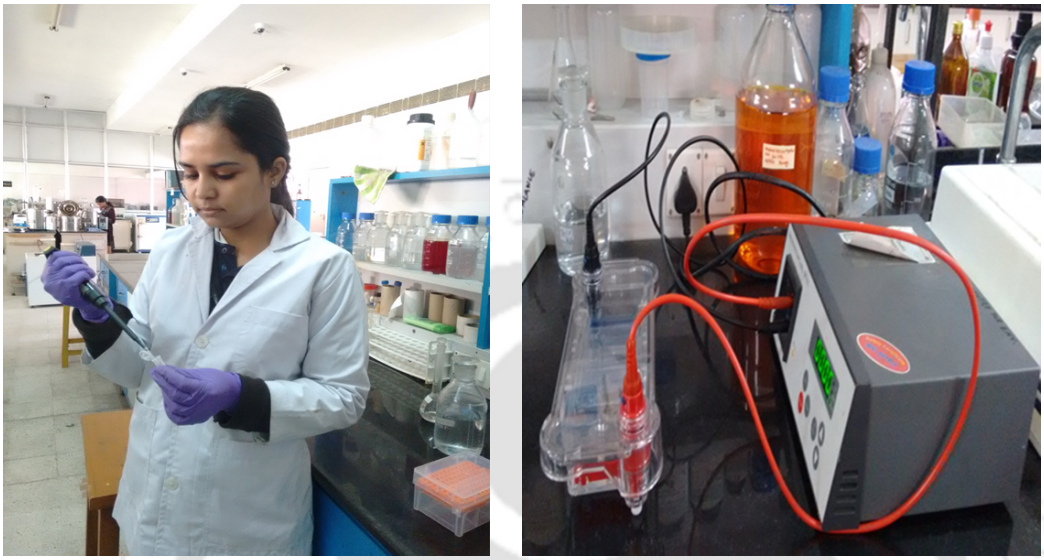


Figure 3.6: PCR amplification and agarose gel electrophoresis experimentation

3.4 PHASE III: Biosorption of Lead and Cadmium using *Bacillus badius* AK Strain Isolated from Compost of Water Hyacinth

3.4.1 Trace Metal Pb(II) Analysis of Water Hyacinth Compost

In order to investigate the amount of lead metal present in the compost from which the isolates were obtained the total metal concentration and the different fractions like water soluble, DTPA and leachable (TCLP) were determined. For all the tests dried sample of compost mass was required. About 800 g of wet compost sample was collected every 4th day starting from 0th day to 20th day was dried at 105°C for 24 h then it was subjected to grinding and was sieved using 75 μm sieve to obtain homogenous sample. Further the procedures were followed as described by Singh and Kalamdhad (2013a).

3.4.2 Selection of Microbe for Metal Removal Study

Microbial growth in an optimized media was selected as the parameter of comparison and the microbe showing highest optical density was selected after incubation time of 24-48 h. The optimized media was taken in 250 mL Erlenmeyer flask and each isolate was inoculated at the same time in separate flask. The optical density at 600 nm was checked for every hour till first 24 h and then after every 6 h and all the readings were plotted to find the microbe with maximum Optical Density.

3.4.3 Metal Removal Study by Living (Non-Pretreated) Bacterial Biomass

3.4.3.1 Effect of Heavy Metal on Bacterial Growth

Pb(II) stock solution was prepared by dissolving appropriate amount of $Pb(NO_3)_2$ for the concentration of 1000 mg/L. Pb-glucose minimal media (selected as optimum media for bacterial growth from previous study) of 100 mg/L concentration was prepared by mixing glucose minimal media ingredients with appropriate amount of Pb(II) stock solution. *Bacillus badius* AK bacterial culture was grown in glucose Minimal media for 24 h. After calculating OD and its highest equivalent value of CFU/mL, 20 mL of the culture was inoculated in Pb-glucose minimal media, the mixture was incubated on rotary shaker at 150 rpm, 30°C. After 1, 2, 3, 6 and 24 h of inoculation, sampling of 20 mL was done in oakridge tube, which was centrifuged at 4000 rpm for 15 min, supernatant was collected for metal analysis. Simultaneously, duplicate sample was used to determine OD at 600 nm, residual glucose concentration was measured by centrifuging the same sample. Second replicate was used to detect the colony forming unit (CFU/mL), spread plating was performed to

identify the corresponding microbial colonies, pH was measured using pH strip (Himedia). Specific growth rate (μ) was estimated by plotting ln(X) vs time curve, The slope equals to specific growth rate. Maximum specific growth rate (μ_{max}) and saturation constant (K_s), (S) is the substrate concentration, X is the biomass concentration, Monod's growth model was fitted according to the Eq. 3.6.

$$\mu = \frac{\mu_{\max}(S)}{K_s + S} \tag{3.6}$$

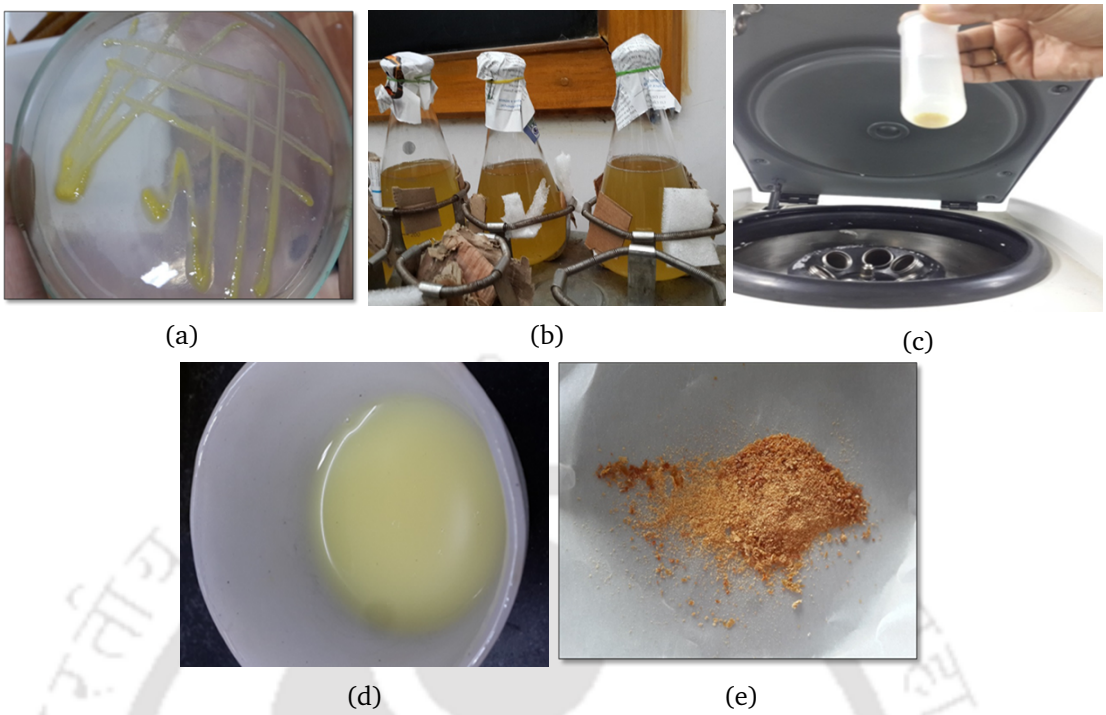
3.4.3.2 Batch Biosorption Studies

In order to have a proper analysis, the value of pH at which metal precipitates in media broth was studied. Initial concentration of Pb(II) was kept same i.e. 100 mg/L, samples were collected at different pH and metal concentration was examined on AAS. To examine the impact of the various factors influencing biosorption, the experiments were performed at temperature of 30°C and pH 4, by incubating for 24 h at 150 rpm. The inoculum was maintained as 20 mL from culture broth. Only the target parameter was varied for each of the test. For pH, tests were performed at varying pH values of 3.5, 4, 4.5, 5 and 5.5. Effect of rotational speed was examined at 80, 100, 120, 150, and 180 rpm, temperature was varied as 20, 25, 30, 35 and 40°C, different initial metal Pb(II) concentration such as 50, 100,150, 200 and 250 mg/L were investigated, inoculum size of 5, 10, 15, 20 and 30 mL from culture broth corresponding to its CFU/mL were used to study its effect on metal removal.

3.4.4 Metal Removal Study by Dried (Pretreated) Bacterial Biomass

1. **Preparation of the Bacterial adsorbent:** *Bacillus badius* AK (KP216715), isolated in previous study from water hyacinth compost was used in this study. The bacterium was cultured in agitated glucose minimal media at 28°C. Cycloheximide (Himedia) (0.2 g/L) was added to prevent any fungal contamination. After 24 h of maximum growth, the bacterium was separated from media by centrifuging at 5000 rpm. Pellets were washed with saline water and phosphate buffer to wash away the media, and then Lyophilized. The powdered form of dried biomass was used for the study as shown in Figure 3.7.

2. **Preparation of lead stock solution:** Lead metal stock of 1000 mg/L was prepared by dissolving lead nitrate ($Pb(NO_3)_2$) salt in distilled water. The stock was allowed to stand for 24 h before being used. Pb(II) solutions of different concentrations were ob-



(a) (b) (c)

(d) (e)

Figure 3.7: (a) Bacterial strain *Bacillus badius* AK on a petri plate. (b) Bacterial growth in glucose minimal media (c) Centrifugation of bacterial biomass (d) Pellets collected after centrifugation (e) Lyophilized bacterial biomass

tained by diluting the stock solutions. Standards required for Atomic Absorption Spectroscopy (AAS) were prepared from pure Certipur Lead standard solution (MERCK). To adjust the pH, 0.1N HCl and NaOH solutions were used and then not further controlled.

3. **Preparation of Cadmium stock solution:** Cadmium metal stock solution of 1000 mg/L was prepared by dissolving 2.74 g of cadmium nitrate ($Cd(NO)_3$) salt in 1 litre of distilled water. The stock was allowed to stand for 24 h before being used. Cd(II) solutions of different concentrations were obtained by required dilution of the stock solution. Standards required for Atomic Absorption Spectroscopy (AAS) were prepared from pure Certipur Cadmium standard solution (MERCK). The 0.1N HCl and NaOH solutions were used for pH adjustment.

3.4.5 Batch biosorption study of Pb(II) and Cd(II)

A batch equilibrium method was used to determine the adsorption of Pb(II) and Cd(II) respectively with dry biomass of *Bacillus badius* AK. For this purpose a set of 250 mL Erlenmeyer flasks containing 50 mL of the metal solution with desired initial concentration were used. About 100 mg of the bacterial dry biomass was added in each flask by varying

the required parameter at room temperature. The flasks were then transferred to a rotary shaker and agitated at 150 rpm. After the desired time interval, flasks were taken out and the contents were poured into a 100 mL centrifuge tubes and centrifuged at 7500 rpm for 10 min. The supernatant were then filtered using Whatman 42 no. filter paper. The samples were stored in specimen tubes and supernatants were analysed for residual concentration on AAS. The capacity of the adsorbent was calculated using the following formula (Eq. 3.7)

$$q_e = \frac{(C_i - C_f)\, V}{M} \tag{3.7}$$

Where q_e is the amount of metal uptake by the biomass adsorbent (mg/g) in equilibrium, C_i and C_f are the initial and final metal ion concentration in solution respectively (mg/L), V is the volume of the metal solution (L), M is the mass of biosorbent used (g).

3.4.5.1 Optimization of Factors Affecting Batch Biosorption Experiments for Pb(II) and Cd(II)

To examine the effects of various parameters affecting the Pb(II) and Cd(II) biosorption ability of the biomass, the above mentioned procedure was followed in all the experiments, unless otherwise mentioned.

1. **Effect of pH:** Tests were conducted by adjusting the pH of the solution at 3, 4, 5 and 6 for Pb(II) and 4, 5, 6, 7, 8 for Cd(II). It was not possible to carry out the biosorption of Pb(II) at pH >6 due to precipitation of the metals as the hydroxides, which makes interpretation of the results difficult. Whereas for biosorption of Cd(II), pH 7 was the critical value of precipitation. The initial biomass concentration was 2 g/L and metal concentration was 100 mg/L and the rotating speed was 150 rpm at room temperature.

2. **Effect of temperature and thermodynamics studies:** Harvested biomass (2 g/L) of *Bacillus badius* AK was exposed to the metal solutions (50 mL, 100 mg/L concentration) in conical flasks incubated at different temperatures ranging from 20-50°C in a shaking incubator at a speed of 150 rpm for 2.5 h. Metal adsorption ability of biomass at varying temperatures was determined by estimating residual metal concentrations in different solutions.

3. **Effect of the contact time:** The optimum dosage and pH were maintained at an initial metal concentration of 100 mg/L and observations were made at every 15 min in the beginning and then after every 30 min for 3 h.

4. **Effect of initial biomass concentration:** To study the effect of biomass concentration

the pH was kept constant at 5 along with the other parameters, only the initial biomass dosage was varied as 0.5, 1, 1.5, 2, 3 and 4 g/L.

5. **Effect of initial metal concentration:** In this case the initial metal concentration was varied as 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L. The tests were carried out at an initial biomass dosage of 2 g/L and a rotational speed of 150 rpm at a temperature of 40°C. All the experiments were performed in triplicate, yielding an experimental error of less than 1%.

3.4.6 Adsorption Kinetics

For the purpose of investigating the mechanism involved in biosorption onto the living (non-pretreated) and dried (pretreated) biomass of *Bacillus badius* AK, as well as potential rate of controlling steps that include mass transport and chemical reaction process, Lagergren pseudo first and second order kinetics models were tested to fit the kinetics experimental data.

For kinetics and isotherm study, the experiment was performed at optimum conditions of temperature, pH, rotational speed and biomass dosage in each case Pb(II) and Cd(II) biosorption. For biosorption study by living (non-pretreated) biomass the inoculum was maintained as 20 mL of culture broth with CFU/mL of 1.5×10^{14} (equivalent to 12.5 mg dry mass). Sampling was done using oakridge tube for centrifuging, the supernatant was collected and stored for metal analysis. The Lagergren pseudo-first order and second-order model were used to determine the order of reaction and value of K_1 was computed using Matlab software. The modeling of pseudo first order kinetics was done by the method of least squares or the Fujimoto method as described in Wastewater Engineering (Metcalf et al., 2003).The least-squares method involves fitting a curve through a set of data points, so that the sum of the squares of the residuals (the difference between the observed value and the value of the fitted curve) must be a minimum. Lagergren linear form of pseudo first and second order kinetics is expressed in following equation (Eq. 3.8) and (Eq. 3.9) respectively (Li et al., 2010; Çolak et al., 2011).

$$\log\left(q_e - q_t\right) = \log q_e - \frac{k_1}{2.303}t \tag{3.8}$$

$$\frac{1}{q_t} = \frac{t}{q_e} + \frac{1}{k_2 q_e^2} \tag{3.9}$$

Where q_e and q_t (mg/g) are the adsorption capacities at equilibrium and time t (min) respectively, k_1 (1/min) is the pseudo-first-order rate constant for the kinetic model, and k_2

(g/mg min) is rate constant for pseudo-second-order equation. In order to select the best fit kinetic model, Chi-square (χ^2) and Root mean square error (RSME) test were done (Eq. 3.10 and Eq. 3.11).

$$\chi^2 = \frac{\sum (q_t - q_{tm})^2}{q_{tm}} \tag{3.10}$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} (q_{ti} - q_{tmi})^2}{n}} \tag{3.11}$$

Where q_t and q_m (meq/g) are metal sorption capacity at time t calculated using experimental data and model, respectively.

3.4.7 Adsorption Isotherms

The adsorptive metal uptake can be quantitatively evaluated by experimental equilibrium isotherms (Puranik and Paknikar, 1997; Namasivayam and Kavitha, 2002). The concentration of adsorbate in liquid phase and its concentration in solid phase are examined at constant temperature, to determine the equilibrium relationship. There are two widely accepted and linearized adsorption isotherm models used in the literature, namely Langmuir and Freundlich. As represented in Eq. 4 and Eq. 5 respectively. Langmuir adsorption isotherm assumes the uniform or monolayer coverage of the adsorbate on the adsorbent's surface. Where, all the adsorprion sites are identical. This provides an idea about the capacity of uptake, reflecting the equilibrium process behaviour (Langmuir, 1918; Duffus, 2002; Mohan et al., 2006; Febrianto et al., 2009).

$$q_e = \frac{q_{\max} K_L C_e}{1 + K_L C_e} \tag{3.12}$$

Where C_e (mg/L) is the equilibrium concentration of metal in solution, q_e (mg/g) the amount of metal adsorbed on the adsorbent at equilibrium, q_{max} (mg/g), the monolayer capacity of biosorbent, K_L is the Langmuir constant, it relates to the capacity and energy of the adsorption.

The Freundlich model is based on the relationship between the metal uptake capacity q_e (mg/g) of adsorbent, and the residual (equilibrium) metal ion concentration Ce (mg/L). It assumes the sorbent to have a surface with a non-uniform distribution of sorption heat.

$$q_e = K_F C_e^{1/n} \tag{3.13}$$

Where q_e (mg/g) is the concentration of metal ion on the biosorbent at equilibrium, C_e (mg/L) is the metal ion concentration in solution at equilibrium. K_F, the Freundlich constant and $1/n$ is the intensity of adsorption.

3.4.8 Temperature and Thermodynamics Study

The temperature was varied between 20-50°C by keeping the samples in a shaking incubator. The pH was adjusted at 5, initial biomass dosage of 2 g/L, initial metal concentration of 100 mg/L and rotational speed of 150 rpm for 3 h. To determine the thermodynamic feasibility and spontaneous nature of the adsorption process, thermodynamic parameters such as free energy change, enthalpy change and entropy change are calculated (Namasivayam and Kavitha, 2002)(Namasivayam and Kavitha, 2002). The free energy change can be related with enthalpy and entropy change by the following equation.

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \tag{3.14}$$

The following linear forms of equations can be used to determine the thermodynamic parameters from the experimental data (Goswami and Purkait, 2012).

$$\log \frac{q_e m}{C_e} = \frac{\Delta S^\circ}{2.303R} + \frac{-\Delta H^\circ}{2.303RT} \tag{3.15}$$

For unit adsorbent mass the equation becomes

$$\log \frac{q_e}{C_e} = \frac{\Delta S^\circ}{2.303R} + \frac{-\Delta H^\circ}{2.303RT} \tag{3.16}$$

Where q_e is the amount of dye adsorbed by unit mass of adsorbent (mg/g), C_e is the equilibrium concentration (mg/L), m is the adsorbent mass (g/L), *R* is the gas constant and *T* is temperature in Kelvin. q_e/Ce is called the adsorption affinity. Enthalpy of adsorption (ΔH°) and entropy of adsorption (ΔS°) can be obtained from the plot of $\log(q_e/Ce)$ vs 1/T. The value of Gibbs free energy (ΔG°) then can be calculated from Eq. 3.14. The positive value of enthalpy of adsorption (ΔH°) will indicate endothermic behavior of the process and negative value will indicate exothermic behaviour of the process. The positive value of Gibbs free energy (ΔG°) will indicate non-spontaneous adsorption process and negative value will indicate spontaneous adsorption process.

Figure 3.8: Biosorption study in a shaking incubator to maintain optimum conditions

3.4.9 Adsorption Mechanism

According to some generally expressed views, adsorption process can be described by the following four consecutive stages. (a) transport of adsorbate in the bulk solution (b) diffusion across the film surrounding the adsorbent particles (surface region diffusion) (c) migration of the adsorbate within the pores of the adsorbent (intraparticle diffusion) (d) adsorption or desorption on the solid surface viewed as a kind of chemical reaction (Plazinski et al., 2009). Determination of adsorption mechanism is required for design purposes and the intraparticle model is commonly used for identifying the adsorption mechanism. Intraparticle diffusion is investigated by Morris-Weber equation and is written as

$$q_t = k_d t^{0.5} + C \tag{3.17}$$

Where, q_t is the amount of metal adsorbed at time t (mg/g), k_d is the rate constant for intraparticle transport/intraparticle diffusion parameter (mg/g.min$^{0.5}$), t$^{0.5}$ is square root of time and C is the thickness of the boundary layer (mg/g).

3.4.10 Desorption, Recovery and Reuse

Biosorption study was performed by using *Bacillus badius* AK against Pb(II) solutions with an initial metal concentration of 100 mg/L at pH 5, for 2.5 h at rotational speed of 150 rpm. The solution was then centrifuged at 6000 rpm for 15 min to separate the biomass

from the solution, the supernatant was preserved to check the final metal concentration. The pellets were then poured in a crucible for drying at 65°C for 20 h. The dried biomass was then used for desorption.

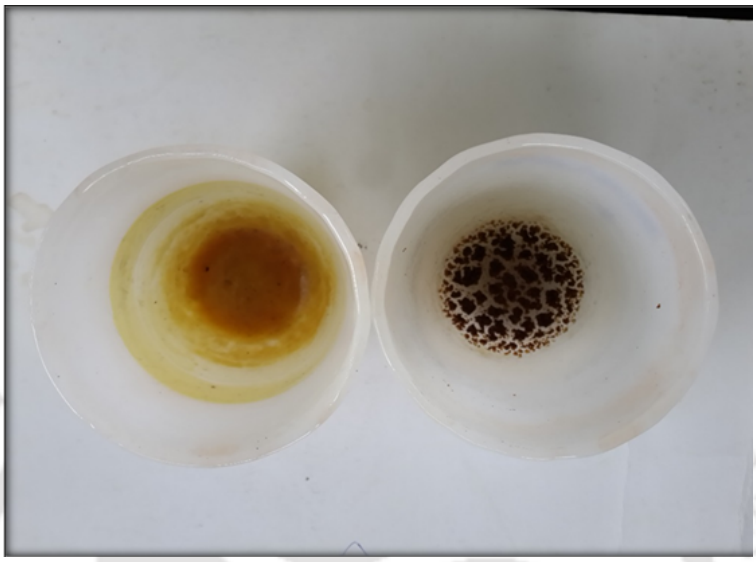


Figure 3.9: Dried biomass before and after biosoprtion

Desorption was done using 0.1M EDTA, HCl, CH_3COOH, HNO_3, H_2SO_4 and distilled water. Desorption was carried out with 50 mL of desorbing solutions for 3 h initially. The samples were then centrifuged and filtered. The final effluent concentration was observed. The filtrate was again recovered and kept for drying for 24 h. Biosorption was again carried out at optimum conditions using the dried sample to find out efficiency after reuse.

3.4.11 Initial Characterisation of Biomass

To find out the characteristics of the biosorbent, different spectroscopic analysis were carried out with the sample before and after biosorption. In order to find out the chemical characteristics and functional groups of the biosorbent before and after biosorption, FTIR spectroscopy was performed. The spectra were recorded in a Fourier transform infrared spectrometer with the samples prepared as KBr discs. All spectra were plotted using the same scale on the transmittance axis. The crystalline structure was determined using XRD technique since it provides the most definitive structural information and interatomic distances.

The surface structure of biosorbent was analysed by Field Emission Electron Microscope (FESEM) coupled with Energy Dispersive X-Ray Analysis (EDX). The acceleration voltage was kept at 3 kV and the microprobe was focussed at different magnification. Biomass samples before and after biosorption were mounted on a stainless steel slab with a double

stick carbon tape followed by coating with a thin layer of gold under vacuum to increase the electron conduction and to improve the quality of the micrographs. The EDX was done under a constant voltage of 20 kV.

The basic elements of any biomass are carbon, hydrogen, nitrogen, sulphur and oxygen was performed by CHNS analysis. CHNS analysis was used to find the carbon present in dry bacterial biomass. Before analysis samples were kept in oven at 105°C for 24 h to remove the moisture. The samples were sent to Guwahati Biotech Park, IIT Guwahati, Assam for analysis.

Brunauer Emmett-Teller (BET) analysis or BET surface area, pore volume and pore size distribution of the sample were investigated by using a computer controlled automated porosimeter. Nitrogen was used as the cold bath (77K) and a classical adsorption model BET theory was used for the determination of the surface area (Sun et al., 2007).

Bulk Density or apparent density was determined as follows: A 10 mL cylinder was filled to a specified volume with dry bacterial biomass that had been dried in an oven at 80°C for 24h (Ahmed and Dhedan, 2012). The bulk density was then calculated as follows:

$$\text{Bulk density} = \frac{W_c}{V_c} \tag{3.18}$$

Where W_c (g) is the weight of dried bacterial biomass and V_c (mL) is cylinder volume packed with dried biomass.

3.4.12 Spectroscopic Analysis

To find out the characteristics of the biosorbent different analysis were carried out for the sample before and after biosorption. In order to find out the chemical characteristics and functional groups of the biosorbent before and after biosorption was done by FTIR spectroscopy. The spectra were recorded in a Fourier transform infrared spectrometer with the samples prepared as KBr discs. All spectra were plotted using the same scale on the transmittance axis. The crystalline structure was determined using XRD technique since it provides the most definitive structural information and interatomic distances. The surface structure of biosorbent was analysed by Field Emission Electron Microscope (FESEM) coupled with Energy Dispersive X-Ray Analysis (EDX). The acceleration voltage was kept at 3 kV and the microprobe was focussed at different magnification. Unloaded and metal-loaded biomass samples were mounted on a stainless steel slab with a double stick carbon tape followed by coating with a thin layer of gold under vacuum to increase the electron conduction and to improve the quality of the micrographs. The EDX was done under a constant voltage of 20 kV and the microprobe was focussed at magnification of 1000×.

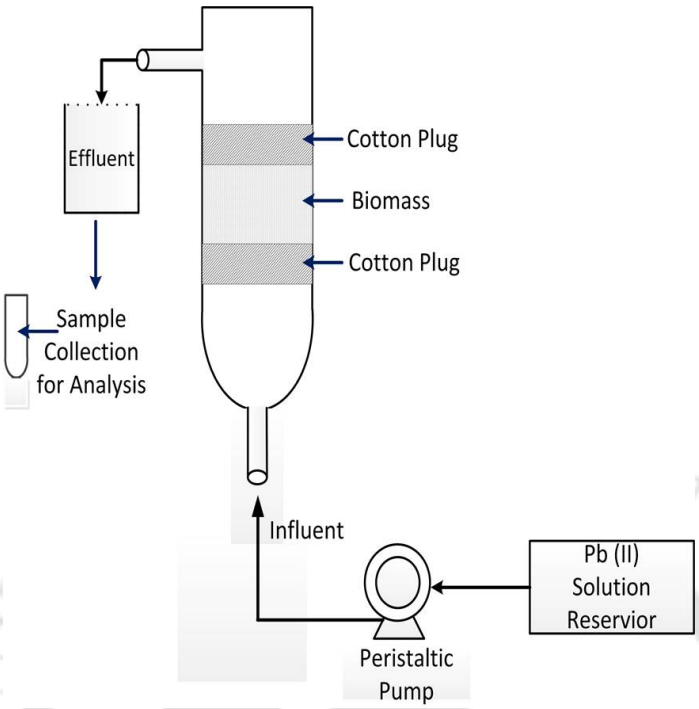
3.4.13 Actual Water Biosorption Experiments

Figure 3.10: Water sample being collected from a tubewell at Adabari, Guwahati

Actual water from a previously known contaminated site was used to check the applicability of the biomass (Fig. 3.10). The pH was adjusted to 5 and the tests were carried out under optimum conditions. Initial characteristics of the sample were found out and the lead concentration was increased to 5, 10, 15 and 40 mg/L since the initial concentration was less. The residual concentration after biosorption was then determined using AAS.

3.4.14 Column Performance

Batch process data has several limitations to predict the performances in practical situation such as actual industrial wastewater plant where continuous reactors are used instead of batch reactors. In batch mode adsorption process is analyzed by adsorption isotherms as the process attains equilibrium. But in fixed bed column mode operation, the adsorption zone keeps moving through the bed and the fluid and the solid phase in that zone are never able to achieve equilibrium (Fig, 3.11). In the present work, dried biomass of *Bacillus badius* AK was used for removal of Pb(II) in continuous mode. The experiments were performed by pumping the Pb(II) solution in a up-flow mode using a peristaltic pump at room temperature (25°C), Pb(II) concentration, influent pH and volumetric flow rate were kept constant at 100 mg/L, 5 and 1 mL/min respectively. Columns of 1 cm diameter were used for different biomass bed depths.



(a)

(b)

Figure 3.11: (a) Schematic diagram of column setup (b) Pictorial view of column setup

Adsorption efficiency by column mode depends on flow rate, bed height, influent adsorbate initial concentration and influent pH. Generally the performance of the continuous

column mode operation is usually described through the concept of breakthrough curve which is the typical 'S' shape profile of exit concentration (C) as a function of time (t) or throughput volume (V_t). Throughput volume is calculated as follows:

$$V_t = Qt \tag{3.19}$$

Where, Q is the volumetric flow rate (mL/min). Breakthrough is defined a phenomenon when concentration of adsorbate from column is 0.1% , 50% or threshold limit of disposal in environment (Gupta et al., 2007; Kumar and Gayathri, 2009; Vimala et al., 2011) of influent concentration. The column is generally considered exhausted when the effluent concentration remains same for long period close to the influent concentration. In the present study exhaustion was considered when effluent metal concentration reached 50% (50 mg/L) of the influent concentration. The area below the breakthrough curve represents mass of metal which is not removed (M) and was calculated using following equation

$$M = \sum[(V_{n+1} - V_n)(C_{n+1} - C_n)/2] \tag{3.20}$$

Where, Vn = throughput volume at nth reading (L), V$_{n+1}$ = throughput volume at (n+1)th reading (L), C$_n$ = effluent adsorbate concentration at nth reading (mg/L) and C$_{n+1}$ = effluent adsorbate concentration at (n+1)th reading (mg/L). Influent adsorbate load (I) was calculated from throughput volume (V_E) at column exhaustion and influent adsorbate concentration (C$_0$) according to (Eq.3.21).

$$\text{Influent adsorbate load (mg)} = I = (V_E \times C_o) \tag{3.21}$$

Mass of the adsorbent removed (M_r) was calculated from difference of influent adsorbate load (I) and mass of adsorbate not removed (M) from (Eq.3.22)

$$\text{Mass of adsorbate removed (mg)} = M_r = (1 - M) \tag{3.22}$$

Adsorbate uptake by adsorbent was calculated using equation 3.7. Adsorbate uptake rate by adsorbent (q_e) = Mr (mg)/ weight of adsorbent (g).

1. **Bed depth service time (BDST) model** There have been several models for predicting the adsorption in lab-scale column studies for designing of the pilot scale columns (Malkoc et al., 2006; Kumar and Gayathri, 2009). Bed depth service time (BDST) is a simple and one of the most applied model for predicting the relationship between bed depth (Z) and service time (t_s) in terms of concentrations and other adsorp-

tion parameters, based on Bohart-Adams equation (Bohart and Adams, 1920) . The Bohart-Adams equation can be represented as:

$$\ln\left(\frac{C_o}{C_b-1}\right) = ln\left(e^{k_{ads}N_o(Z/u\,)}-1\right) - k_{ads}C_o t_s \tag{3.23}$$

Hutchins (1973) modified Bohart-Adams equation and proposed a linear relationship between bed depth and service time. [Eq. (3.16)], which requires only three fixed-bed tests to collect the necessary data.

$$t_s = \frac{N_o Z}{C_o u} - \frac{1}{k_{ads}C_o}\ln\left(\frac{C_o}{C_b}-1\right) \tag{3.24}$$

Where t_S is the service time at breakthrough point (h), N_o the dynamic bed capacity (mg/L), Z the packed-bed column depth (cm), u the linear flow rate (cm/h) defined as the ratio of the volumetric flow rate Q (cm^3/h) to the cross-sectional area of the bed A (cm^2), C_o and C_b are, respectively, the influent and the breakthrough adsorbate concentration (mg/L) and k_{ads} is the adsorption rate constant (L/mg h). Critical bed depth (Z_o) gives the minimum depth of adsorbent in the column that would be capable of preventing the adsorbate concentration from exceeding the breakthrough concentration (mg/L). It can be obtained when breakthrough is immediate and calculated by substituting $t_s = 0$ in Eq. 3.25 as shown below:

$$Z_o = \frac{u}{k_{ads}N}\left(\frac{C_o}{C_b}-1\right) \tag{3.25}$$

3.4.15 Statistical Analysis

The results reported are means of three replicates. Repeated measures were treated with analysis of variance (ANOVA) using Statistica software. The objective of the statistical analysis was to determine any significant differences among the parameters analysed for different trials.

3.5 Instruments Used

Table 3.4 shows the various instruments used during investigation and experimental analysis.

Table 3.4: Instruments used during investigation and experimental analysis.

Parameter Analysis	Instrument	Make and Model
Sieving	Sieve	Unique Drawing & Survey emporium
Weighing	Analytical weighing balance	Model: HPB220, Wensar
Moisture content	Class- II High Accuracy	Satwik Scale Industries
pH	μ pH system 361	Model: μpH system 361, M/S Systronics India Ltd.
Electrical conductivity	Conductivity meter VSI-04 Deluxe	VSI Electronics Pvt. Ltd.
Volatile solids	Muffle Furnace	International Commercial Traders
CO_2 evolution	BOD Incubator	International Commercial Traders
OUR	DO meter	Eutech Instrumets Pvt. Ltd.
COD	COD Digester	HACH
BOD	BOD Incubator	International Commercial Traders
Optical density	UV spectrophotometer	Varian, model Cary 50 (Netherland)
Serial dilution	Vortex	SPINIX
Plating, MPN	Laminar flow chamber	CleanAir
Sterilization	Autoclave	Equitron
Metal analysis	AAS	Model: 55 B, M/S spectra AA Varian
Total metals	Digester	Pelican
Optical density	Spectrophotometer	MRC
Morphology	Microscope, FESEM, SEM	Olympus E330-ADU1.2X, Japan, Model: LEO 1430 VP, M/S Carl Zeiss Germany
Surface area analysis	Surface area analyser (BET)	Quantachrome
Centrifugation	Centrifuge	Remi Model: Autosorb-IQ MP
Functional groups	FTIR	Perkin elmer spectrum Version 10.4.3
Column Study	Peristaltic Pump	Model: PP-60 EX 3C, Miclins India, Chennai
Mixing	Shaking incubator	Model: LSI-1005R, DAIHAL Labtech Co. Ltd, Korea

"What you see is that the most outstanding feature of life's history is a constant domination by bacteria."

Stephen Jay Gould

4

Rotary Drum Composting and Microbiology of Water Hyacinth Compost

This chapter investigates the performance of rotary drum composter for the degradation of water hyacinth in combination with bulking agents such as cow dung and sawdust. In addition, the microbial study of best trial of water hyacinth compost was performed along with the isolation and identification of microbes from the water hyacinth compost. The study was conducted in two phases:

1. **Phase I:** Rotary drum composting of water hyacinth using 5 different waste ratio combinations and their physio-chemical, biological and microbial analysis.
2. **Phase II:** Microbial Succession (DNA) analysis and isolation and identification of bacteria from the best trial of water hyacinth compost

4.1 PHASE I: Microbial Population, Stability and Maturity Analysis of Rotary Drum Composting of Water Hyacinth

Composting is used as a treatment method for some wastes which yields a stable and profitable product. The term composting has been defined by (Crawford, 1983) as the incomplete, artificially accelerated decomposition of heterogeneous organic matter by a mixed microbial population in a warm and moist environment. Microbial communities change with various stages of composting. There is an initial temperature rise due to the availability of organics and transition of microbes from mesophilic to thermophilic microflora (Ryckeboer et al., 2003). Under the optimal conditions composting can be divided into four phases (a) mesophilic phase (10-40°C) lasting for few hours to some days, (b) thermophilic phase (42-65°C) lasting for few days, but its duration can vary with the type

of material used, as in case of wood it can last for months also, (c) the second mesophilic phase is where the microbes recolonize the substrate, (d) last is the maturation or curing phase in which micro biota is still present in the compost but with less vigorous activities which can make the compost more mature and stable (Ryckeboer et al., 2003). The microbial communities in whole composting period keep on changing (Hassen et al., 2001; Bhatia et al., 2012). Bacteria, actinomycetes, streptomycetes and fungi are the major contributors of biodegradation. The type and count of these microbes change with the availability of organic matter and prevailing physiochemical conditions. Microbial activity is interdependent on several factors such as O_2 supply, moisture content, temperature, and pH. These factors affect the microbial growth, activities and hence the whole composting process. The quality of compost can be estimated with the relative study of these parameters and microbes in the compost.

Progress has been made in the characterization of some organisms typically found at different stages of composting by counting with plate-count method and isolating microbes (Boulter et al., 2002). Davis et al. (1992b) studied that during all stages of composting of pine bark, bacterial CFU outnumbered fungal CFU. Ma et al. (2003) investigated that during composting the sequence of microbial succession was from mesophilic bacteria to thermophilic bacteria and thermophilic actinomycetes during composting of anthracene contaminated soil. A total of 194 fungal species have been isolated from green compost and vermicompost (Anastasi et al., 2005). Partanen et al. (2010) studied bacterial diversity of over 2000 different phylotypes in municipal organic waste composting. Bhatia et al. (2012) studied that a majority of bacterial species isolated from compost includes *Bacillus* sp., *Pseudomonas* sp. and *Entrobacter* sp., whereas *Aspergillus* and *Rhizopus* species are the fungal communities in windrow and rotary drum composting of vegetable waste. Studies have been carried out on the microorganisms involved in composting of a various types of solid wastes such as municipal sewage sludge and wheat straw (Ryckeboer et al., 2003), pine bark (Davis et al., 1992a), pulp and paper-mill solids (Atkinson et al., 1997), municipal solid waste (Hassen et al., 2001), garden waste (Vítězová et al., 2013), anthracene-spiked soil mixed with kitchen waste (Ma et al., 2003) and municipal solid waste (Raut et al., 2008). However, there is no study available on microbial dynamics of water hyacinth composting.

Water hyacinth is known to be one of the world's worst and noxious aquatic weed growing over a wide variety of wetlands such as lakes, streams, ponds, waterways, ditches, and backwater areas (Holm et al., 1991). It forms dense mat which interferes with navigation, recreation, irrigation, and power generation (Dhal et al., 2012). Water hyacinth obtains its nutrients directly from water and has been used in wastewater treatment facilities (Wilson et al., 2006). It has been utilized somewhat effectively as a substitute of bean straw for

animal feed, also as feed for solid-phase fermentation, raw material for making pulp, paper, and paper board and in vermicomposting also. The successful application of rotary drum composting of water hyacinth has been carried out using cow dung and sawdust (Dhal et al., 2012). The composting process has been optimized to yield compost in 20 days with turning frequency of one turn per day. But as composting is a microbial phenomenon the study of microbial dynamics of water hyacinth composting was essential.

In phase I of water hyacinth composting, micro-flora and their relation to abiotic parameters were investigated. Traditional microbial techniques were used for the purpose. As the gases (O_2 and CO_2) concentrations in the composting mass reflect its microbial activity, thus OUR and CO_2 evolution rate were also examined in relation to the variances in microbial community. Heat is generated as the metabolic waste product in compost due to the active microbial participation in converting the organic waste matter into inorganic and humified forms. High temperature supports degradation of defiant lignocellulosic material (Tuomela et al., 2000), so monitoring of temperature in relation to the microbes was also done.

4.1.1 Initial characterization of waste materials

Before feeding the rotary drum composter with the feedstock materials for composting, initial characterization of waste materials was performed. A higher pH value of 8.4 was observed in water hyacinth. Moisture content was also highest in water hyacinth with 87.3% as compared to cow dung and sawdust. Soluble BOD and Soluble COD of water hyacinth was 795 g/kg and 864 g/kg respectively, which was higher than other waste materials. Highest oxygen uptake rate of 5.8 mg/g VS/day was observed in cow dung, while OUR remained undetected in sawdust, as no readily degradable organic matter is present in sawdust. Highest count of microbial communities such as mesophilic, spore forming bacteria, actinomycetes, streptomycetes and fungi was also observed in water hyacinth and cow dung as shown in Table 4.1.

4.1.2 Physio-chemical analysis of compost

- **Temperature**

Periodic measurement of the temperature changes in the compost is an important index to measure the microbial activities. It determines the rate at which many of the biological processes take place and thus, it plays a selective role on evolution and succession of the microbiological communities (Hassen et al., 2001). (Figure 4.1) gives the details of the temperature variations throughout the 20 days of composting period. As compared to all trials, trial 3 showed the maximum temperature of 56.50°C. As reported by Singh and

Table 4.1: Composition and initial characterisation of waste materials

Trial/Parameter	Waste Material (kg)		
	Water hyacinth	**Cow dung**	**Sawdust**
EC (ds/m)	8.42 ± 0.02	4.63 ± 0.01	0.45 ± 0.02
Volatile Solids (%)	86 ± 0.02	87.2 ± 0.01	96 ± 0.01
pH	5.94 ± 0.01	7.16 ± 0.03	5.85 ± 0.02
Moisture Content (%)	87.3 ± 2.21	74.76 ± 0.78	43.74 ± 0.43
Soluble BOD (g/kg)	795 ± 20	480 ± 30	150 ± 30
Soluble COD (g/kg)	864 ± 20	648 ± 16	432 ± 20
Oxygen Uptake Rate (mg/g vs /day)	1.89 ± 0.9	5.8 ± 0.7	ND
CO_2 Evolution Rate (mg/g vs /day)	1.6 ± 2.5	1.44 ± 0.7	0.68 ± 0.4
Spore Forming Bacteria (CFU/g)	1.06×10^{8}	9.00×10^{6}	8.00×10^{5}
Mesophilic bacteria (CFU/g)	1.71×10^{12}	1.40×10^{14}	1.32×10^{9}
Actinomycetes (CFU/g)	8.25×10^{9}	1.84×10^{9}	1.24×10^{8}
Streptomycetes (CFU/g)	1.78×10^{7}	7.00×10^{8}	1.04×10^{8}
Fungi (CFU/g)	8.90×10^{9}	4.00×10^{6}	7.80×10^{5}
Total heavy metals (mg/kg dry matter)			
Pb	1120 ± 5	800 ± 4	992 ± 5
Cd	39 ± 7	49 ± 1.2	52 ± 4
Zn	168 ± 3.1	174 ± 2.1	120 ± 2.4
Ni	162 ± 8.9	220 ± 1.7	249 ± 2.4

Note:(mean ± SD, n=3) SD- standard deviation, ND-Not detected.

Kalamdhad (2012) providing optimum carbon to nitrogen ratio by proper combination of waste materials lead to high temperature rise. The rise in temperature started within 24 h of composting and reached its maximum by 4^{th} day, after this it started to stabilize. It has been reported that during the composting process temperatures of 520°C to 600°C was considered to maintain the greatest thermophilic activity (Ryckeboer et al., 2003). The average highest temperature for all the trials was in the range of 50-56.50°C, but the highest temperature in control reached up to 38.60°C only. On analyzing the results by ANOVA, the

variation in temperature varied significantly between the days ($p < 0.05$). This indicated that for proper degradation of organic matter cattle cow dung and sawdust are required along with water hyacinth.

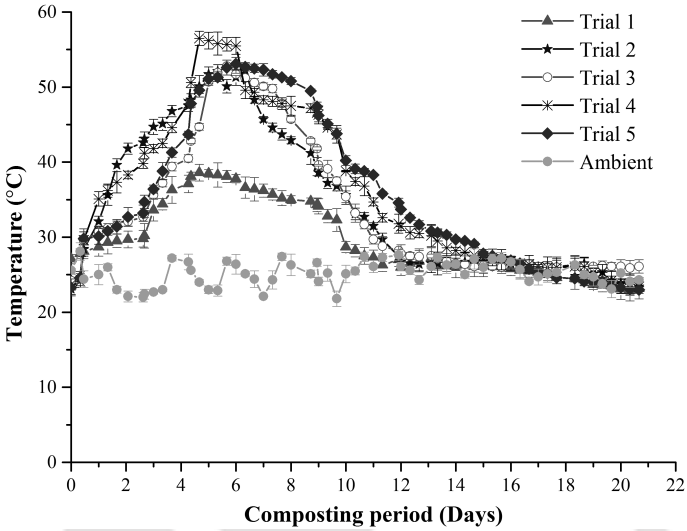


Figure 4.1: Variation in temperature profile during composting period

• **Volatile solids (VS%)**

Volatile solids give an account of the oxidized organic matter when it is heated. Soluble organic material can be easily degraded as being the primary nutrient of microbes (Said-Pullicino et al., 2007). However, the total organic matter in plant material comprises of cellulose, hemicelluloses, reducing sugars and lignin. These are highly biodegradable, but lignocellulose is partially solubilized (almost unchanged) and reorganized to form humus-like substances (López-González et al., 2013). The volatile solids analyzed, reduced from 60 to 48%, 58 to 44%, 67 to 36%, 65 to 40% and 73 to 61% in trials 1, 2, 3, 4 and 5, respectively (Figure 4.2) from 0^{th} to 20^{th} day. On analyzing the results by ANOVA, the decrease in VS% varied significantly between the days ($p < 0.05$). Highest amount of VS reduction of 46% was observed in trial 3, this much reduction showed incomplete degradation. Highest temperature in trial 3 resulted into proper degradation of organic matter as compared to other trials, this resulted into more VS reduction. Due to the presence of complex lignocellulosic material in the available organic matter the reduction of VS requires more time.

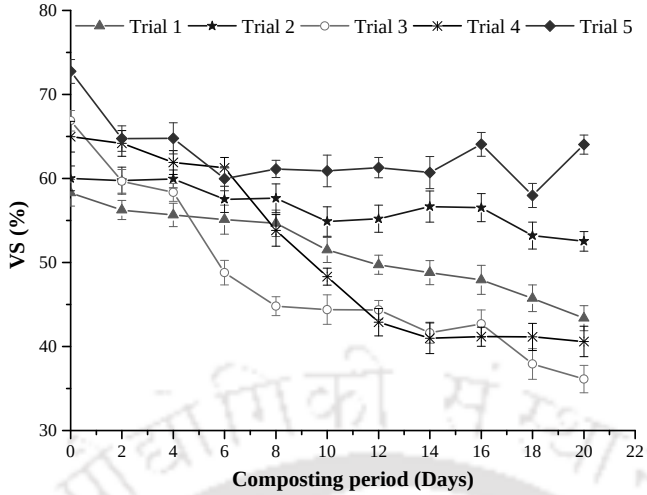


Figure 4.2: Variation in Volatile solids (%) during composting period

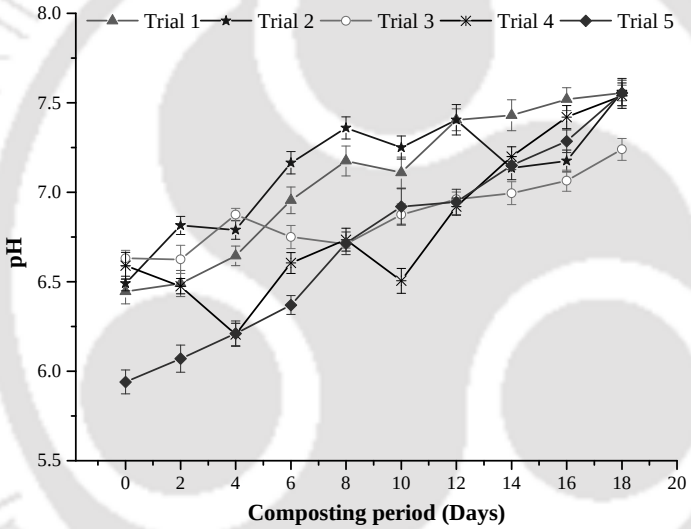


Figure 4.3: Variation in pH during composting period

• **pH**

pH is another factor which greatly influences the composting process by affecting microbial activity. The optimal range for the development of bacteria and fungi is 6.0 to 7.5 and 5.5 to 8.0 respectively (Ryckeboer et al., 2003). The variation in pH changes the availability of hydrogen ions. These hydrogen ions play a vital role in the transport across the microbial membrane and thus, affecting the activities of composting microbes (Plette et al., 1995). A pH range of 6.5 to 8.0 had been reported favourable for microbial growth in wheat straw (Pan and Sen, 2013), pH of 4.0 and 6.5 in food waste and garden clipping composting respectively. However, in present study the initial pH of water hyacinth, cattle (cow) manure and sawdust was observed to be 6.5, 7.2 and 5.9, respectively. The initial

and final pH of all the trials was in the range of 5 to 6.6 and 7 to 7.6, respectively. (Figure 4.3) shows the pH range of all the trials. The ANOVA analysis depicted a significant variance of pH between the trials ($p < 0.05$). The observed pH favored the growth and activity of bacteria, fungi and all other microbes also. All these pH values favored the development of optimum conditions for microbial growth in compost sample.

• **Electrical conductivity (EC)**

Electrical conductivity (EC) is indirect measurement of soluble salts, and is used as chemical indicator of composting (Pan and Sen, 2013). High EC in the final compost of water hyacinth slows down plant rooting and reduce the transportation of water and nutrients into the plants (Singh and Kalamdhad, 2013a). The final EC values of all composts were 5.4, 6.2, 2.9, 4.7 and 9 dS/m in trials 1, 2, 3, 4 and 5, respectively (Figure 4.4). It was observed that the EC value decreased with the progress in composting in all trials except trial 5. Similar values of EC in water hyacinth compost were also monitored by Singh and Kalamdhad (2012). However, 6 dS/m of EC has been reported in sludge composting (Pan and Sen, 2013). The volatilization of ammonia and the precipitation of mineral salts is possible reason for the decrease in EC at the later phase of composting (Dhal et al., 2012). Due to the release of humic substances (which has the capacity to interact with metal ions) reduction of water solubility was observed (Singh and Kalamdhad, 2012), the EC might have decreased. On analyzing the results by ANOVA, EC varied significantly between all the trials ($p < 0.05$). After 20 days, only compost of trial 3 approached the desired EC value. Higher number of microbes in final compost would cure and mature the final compost.

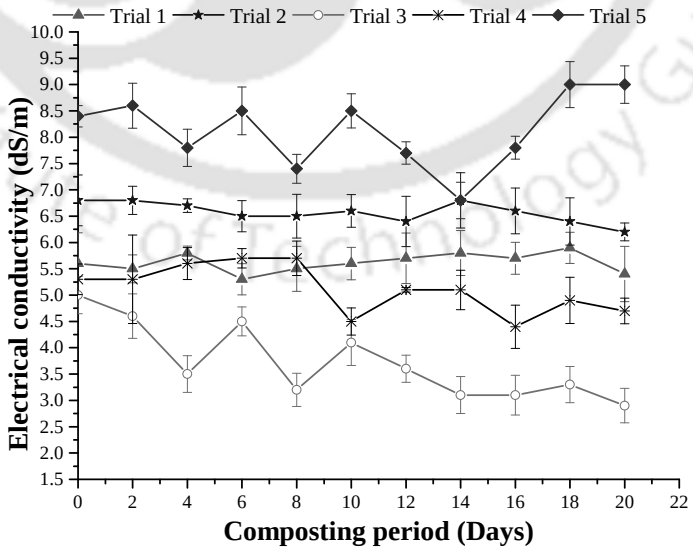


Figure 4.4: Variation in Electrical conductivity during period

4.1.3 Biochemical Parameters

The chemical oxygen demand (COD) test determines the organic content in terms of both biodegradable and non-biodegradable compounds, whereas the biochemical oxygen demand (BOD) test evaluates the biodegradable fraction.

• Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD)

Chemical oxygen demand (COD) is measure of total amount of oxygen required to oxidize all organic matter into carbon dioxide and water. Instead of free dissolved oxygen, chemically bound oxygen in potassium dichromate and sulphuric acid is used to oxidize the organics thereby producing carbon dioxide and water. The COD values reduced down from 0th to 20th day of composting from 6.8 to 3.2, 5.8 to 2.5, 8.2 to 2.1, 6.8 to 2.7 and 8.6 to 4.3 g/kg in trials 1, 2, 3, 4 and 5, respectively (Figure 4.5). A maximum of 73.9% reduction was found in trial 3, depicting high amount of reduction in easily degradable organic matter of the compost. On analyzing the results by ANOVA, a significant variance among all the trails was observed ($p < 0.05$).

Biochemical oxygen demand (BOD) is the amount of dissolved oxygen needed by aerobic microorganisms to break down the organic material in the compost sample over a specific time period (Kalamdhad et al., 2008). High concentrations of BOD in compost can deplete dissolved oxygen in water content of soil and thus have a negative impact on soil structure. The values of BOD changed progressively from 4.3 to 2.2, 4.8 to 2.4, 5.4 to 1.8, 4.0 to 1.6 and 7.9 to 4.2 g/kg in trials 1, 2, 3, 4 and 5, respectively (Figure 4.6) during 20 days of composting. The ANOVA analysis depicted a significant variance among all the trials ($p < 0.05$). The maximum reduction was found about 66.7% in trial 3. This depicted that the easily degradable organic matter has been broken down by the microbes in trial 3 more efficiently than other trials.

BOD/COD ratio depicts the stability of compost as high value of this ratio would indicate the presence of organic matter that can readily degraded (Kalamdhad and Kazmi, 2009). Some toxic organics curbing the microbial activities may also be responsible. Presently it was estimated that the BOD/COD ratio on 0th day of composting was 0.6, 0.8, 0.6, 0.5 and 0.9 which reduced down to 0.7, 0.9, 0.8, 0.6 and 0.9 in trials 1, 2, 3, 4 and 5, respectively. The highest reduction in BOD and COD values was observed in trial 3. This value of BOD/COD ratio in trial 3 reduced to 0.6 which was greater than trial 4 value (0.5), it suggested that the higher microbial activities in trial 3 would have enhanced the BOD value more than trial 4, but observing the respective reduced values of BOD and COD indicates that trial 3 compost is more stable than trial 4. The reduction in COD is

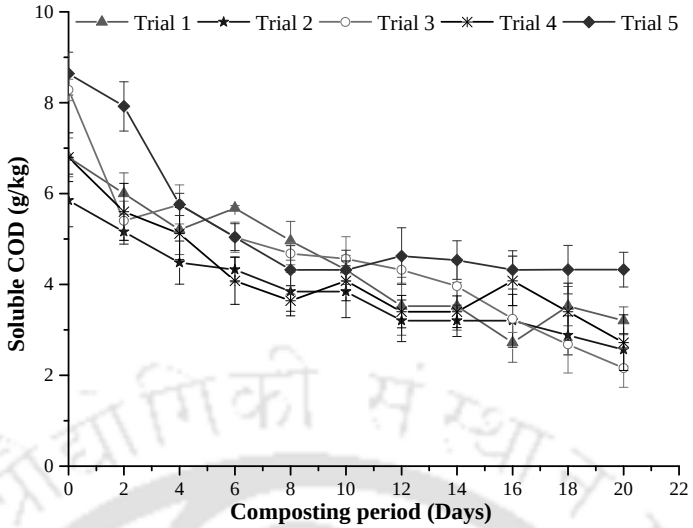


Figure 4.5: Variation in Soluble COD during composting period

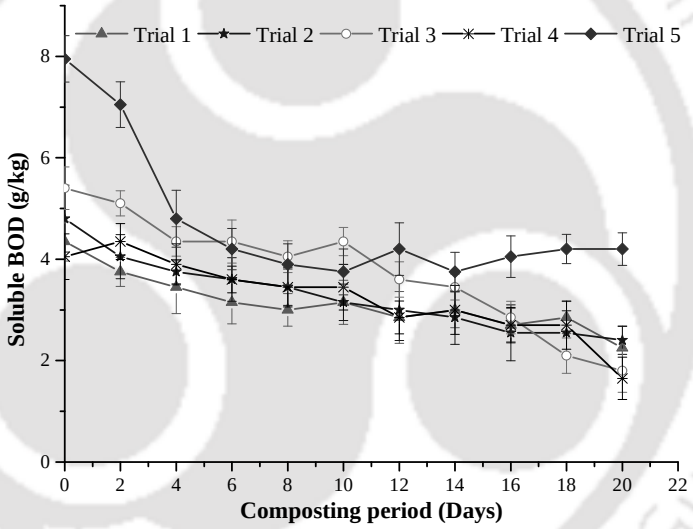


Figure 4.6: Variation in soluble BOD during drum composting period

the indicator of stabilization of compost. During the course of composting, the biological organic content reduces, as a result the emission of carbon dioxide gets reduced, it results into decrease in BOD and COD.

- **Stability of compost**

Stability determines the compost nuisance potential, nitrogen immobilization and phytotoxicity (Kalamdhad et al., 2008). Thus, it can indicate compost quality. The stability of the compost is measured directly by the CO_2 evolution rate and oxygen uptake rate (OUR) during the composting process. CO_2 evolution rate is associated with degradation of volatile

material of compost (Ma et al., 2003), it also gives information about the aerobic respiration occurring in composting and OUR indicates the amount of readily degradable organic matter present in the compost sample (Kalamdhad et al., 2008). The CO_2 evolution rate reduced from 6.48 to 1.31, 6.09 to 0.98, 6.41 to 0.60, 5.99 to 0.76 and 1.62 to 0.58 mg/g VS/day in trials 1, 2, 3, 4, and 5 respectively (Figure 4.7). On analyzing the results by ANOVA, a significant variance among all the trials was observed ($p < 0.05$). A maximum of 90.6% reduction was observed in trial 3, which signifies that compost in trial 3 was most stable. This highest degradation shows that least amount of organic matter remained available to be degraded showing greatest variation of CO_2 evolution rate from 0^{th} till the last 20^{th} day of composting.

OUR will be observed high as the microbial population will feed on the raw material available in the compost sample (Iannotti et al., 1993). The OURs of trial 1, 2, 3, 4 and 5 decreased from the initial values of 7.13 to 4.79, 7.94 to 3.92, 9.40 to 2.00, 8.95 to 3.24 and 1.89 to 1.41 mg/g VS/day, respectively (Figure 4.8). Highest decrease of 78.7% was observed in trial 3. The initially available organic matter amount was higher and also very complex for microbial action, thus required more oxygen for its degradation. On analyzing the results by ANOVA, OUR varied among all the trials ($p < 0.05$). The availability and complexity of the degradable matter decreased during the process, thus oxygen demand reduced too. This stated that the compost was stable enough to be further degraded by the microbes. In present study trial 3 had shown maximum reduction in CO_2 evolution rate and OUR, stating that the compost of trial 3 was most stable. Similar results were also reported by Kalamdhad et al. (2008).

The ratio of CO_2 produced and O_2 consumed gives information about the Respiratory quotient (RQ). It depends upon the composition of organic waste and active microbial communities. For aerobic respiration value of RQ is equal to 1 (Gea et al., 2004). Other than this RQ is affected by factors also like pH, temperature, particle size, organic content, methane and nitrogen oxides emission (He et al., 2001). Authors have reported different RQ values for different wastes, like for municipal solid waste it was in range of 1.02 to 0.95 (Teresa et al., 2004), for paper sludge 0.92 (Atkinson et al., 1997). Other studies have related RQ with temperature also as metabolism of microbes (Nakasaki et al., 1985). In present study RQ values of trial 1, 2, 3, 4 and 5 decreased from 0.91, 0.77, 0.68, 0.67, 0.86 to 0.27, 0.25, 0.30, 0.24 and 0.41 from 0^{th} till last 20^{th} day, it was observed that the RQ values were quite stable in trial 3 from 0th till 8^{th} day, which depicted highest degradation and temperature. Thus, organic matter degradation in 6:3:1 composition was more than any other composition.

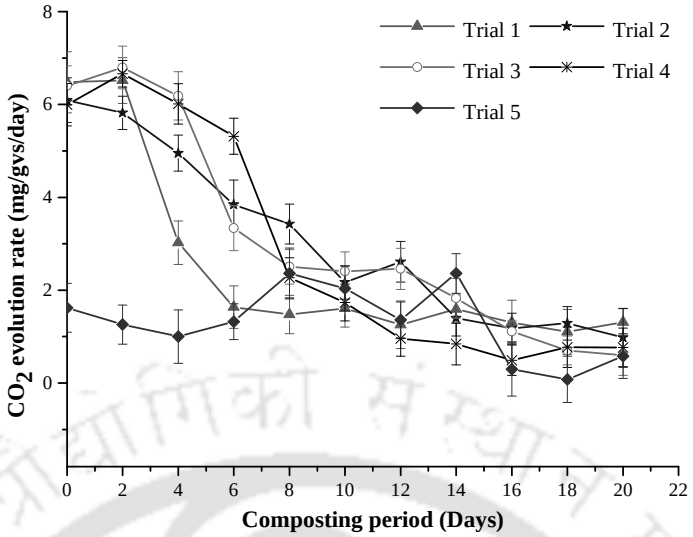


Figure 4.7: Variation in CO_2 evolution rate during composting period

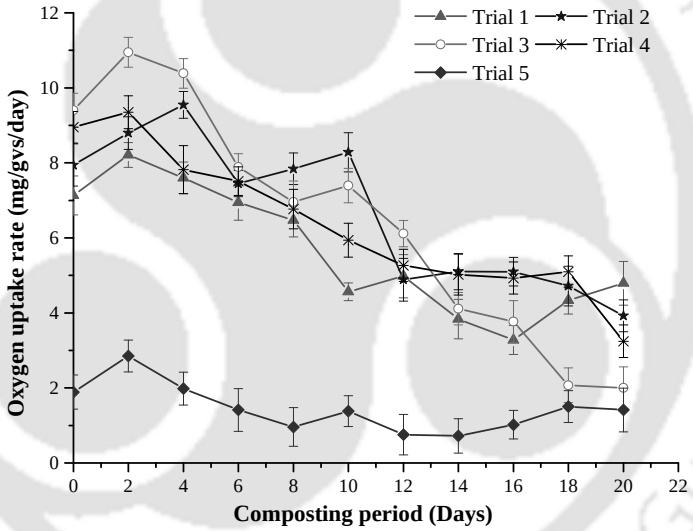


Figure 4.8: Variation in oxygen uptake rate during composting period

4.1.4 Analysis of Microbial community

The waste materials used for composting are full of pathogenic and non pathogenic microorganisms. Various microbes such as, bacteria, actinomycetes, streptomycetes and fungi participate in the degradation of the organic matter. All these microbes have different properties showing different growth pattern and degrading properties releasing respective extracellular enzymes. Thus, all these microbes will show their action at different stages of composting.

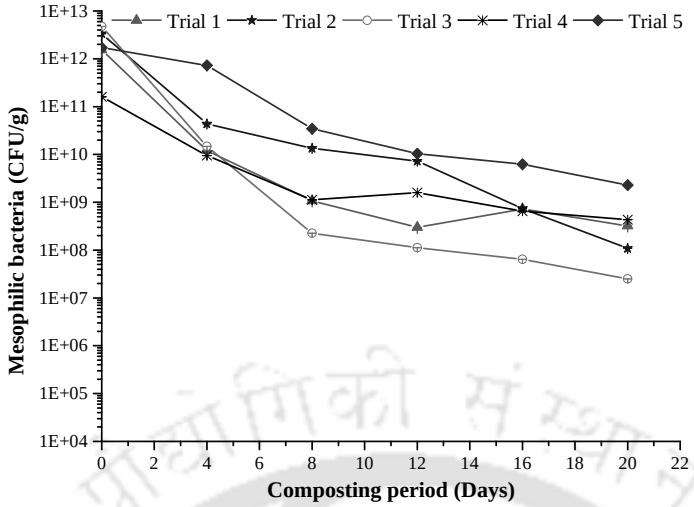


Figure 4.9: Variation in mesophilic bacteria during composting period

• **Mesophilic and spore forming bacteria**

Mesophilic and thermophilic stages of the composting system play an important role in the growth and development of mesophilic as well as thermophilic microbes (Riddech et al., 2002). In first week of composting intense microbial activity and organic matter degradation is reported (Chazirakis et al., 2011), which causes the rise in temperature. Initially the temperature of compost was ambient, but slowly within 24 to 48 h it started rising up. The maximum temperature was recorded up to 56.5°C among all the trials. This rise in temperature occurred till 4^{th} day of the composting period, after this it declined and started getting stabilized. The initial count of the mesophiles was 1.53×10^{12}, 3.36×10^{12}, 4.73×10^{12}, 1.60×10^{11} and 1.71×10^{12} CFU/g in trials 1, 2, 3, 4 and 5, respectively (Figure 4.9). The temperature rise affected the growth and population of the mesophilic microbes and their count declined to 3.21×10^{8}, $1.0^{8} \times 10^{8}$, 2.5×10^{7}, 4.35×10^{8}, 2.3×10^{9} CFU/g in trials 1, 2, 3, 4, and 5, respectively by end of composting period.

With the rise in temperature throughout the composting process the mesophilic bacteria declined and spore forming bacteria were seen proliferating. However, mesophilic bacteria with thermo tolerance property (surviving at 56-60°C) have been reported (Ma et al., 2003), the count of spore forming bacteria on the 0th day of composting in trials 1, 2, 3, 4 and 5 was 5.28×10^{7}, 1.32×10^{8}, 1.80×10^{8}, 1.88×10^{7}, 1.06×10^{8} CFU/g, respectively (Figure 4.10). Gradually after 24 h, when the temperature started rising up the fluctuations in their count appeared. On 4^{th} and 8^{th} sampling day the count of spore forming bacteria was found to be highest among all the trials. On 4^{th} day it was 1.80×10^{9}, 1.73×10^{10}, 3.3×10^{10}, 1.53×10^{9} and 9.83×10^{8} CFU/g in trials 1, 2, 3, 4 and 5, respectively. On the 8^{th} day the count varied as 5.20×10^{9}, 5.21×10^{10}, 7.2×10^{10}, 1.0×10^{9} and 6.83

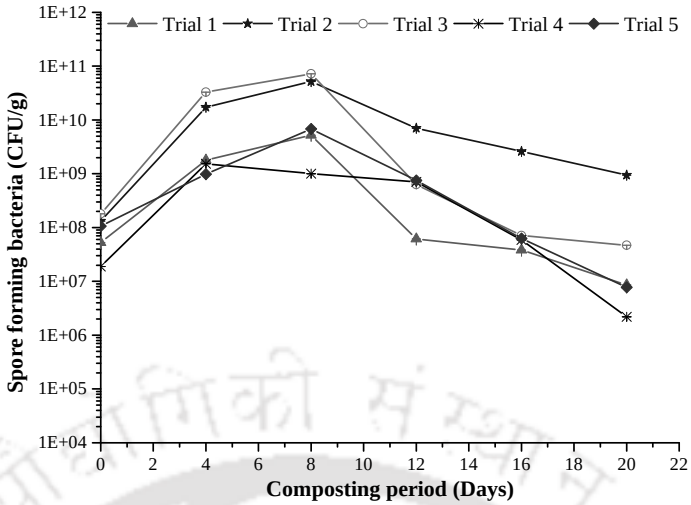


Figure 4.10: Variation in spore forming bacteria during composting period

$\times$ 10^9 CFU/g, respectively. Highest count of spore forming bacteria was observed in trial 3 having the highest temperature of 56.5°C. After 8^{th} day the temperature declined along with the count of spore forming bacteria. The count decreased to 8.60 $\times$ 10^6, 3.4 $\times$ 10^8, 5.7 $\times$ 10^8, 2.2 $\times$ 10^6 and 7.8 $\times$ 10^6 CFU/g in trials 1, 2, 3, 4 and 5, respectively on the 20th day (Figure 4.10). However total thermophilic bacteria (spore forming bacteria) grew fast from 1.3 $\times$ 10^4 CFU/g to 1.0 $\times$ 10^9 CFU/g in biowaste composting (Chroni et al., 2009). On analyzing the results by ANOVA, a significant variance among all the trails was observed for both mesophilic and spore forming bacteria ($p < 0.05$).

The carbon content of the organic matter degrades to be released in form of CO_2. The temperature rises as the microbes were highly metabolically active, thus giving the indication about the level of maturity of compost. In comparison to all the trials, trial 3 showed the maximum decrease in mesophilic bacterial count, as maximum temperature was depicted among all the trials. Trial 3 had the maximum count of mesophilic and spore forming bacteria among all the trials. This signified better degradation of the organic material. Thus, the present study stated that bacterial count widely relates with the changes in temperature and the availability and type of organic matter.

• Actinomycetes and streptomycetes

Actinomycete cellulases being secreted by actinomycetes are inducible extracellular enzymes. Actinomycetes attack cellulose in much the similar way as fungal hydrolytic cellulose (McCarthy, 1987). Actinomycetes have also been reported in lignocelluloses degradation. Some species appear during the thermophilic phase, and some become an important part during the cooler maturing phase. These microbes are mostly seen towards the end of

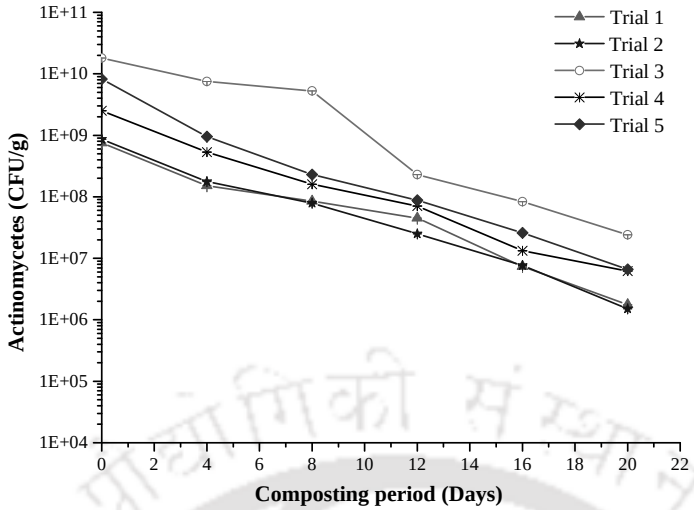


Figure 4.11: Variation of actinomycetes during composting period

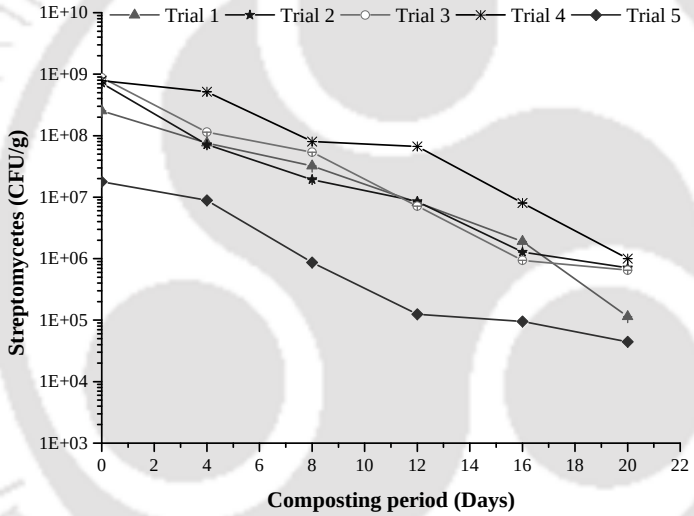


Figure 4.12: Variation of streptomycetes during composting period

composting process. Municipal solid waste has 51.1% of actinomycetes concentration (Rebollido et al., 2008). Whereas in present study the number of actinomycetes varied from 0th till last 20th day of composting. The initial count reduced from 7.5×10^8 to 1.76×10^6, 8.6×10^8 to 1.5×10^6, 1.8×1^{10} to 2.4×10^7, 2.5×10^9 to 6.2×10^6 and 8.25×10^9 to 6.61×10^6 CFU/g (Figure 4.11) in trials 1, 2, 3, 4 and 5, respectively during the composting period. Their count was found highest in trial 3 as compared to other trials, which denotes better degradation of the organic matter and hence more cured or mature compost.

Streptomycetes are also considered to play an important role in the degradation of recalcitrant macromolecules. They are responsible for production of antibiotics like streptomycin.

In fact, over two thirds of the antibiotics used today are derived from one of the 500 species of streptomyces. The populations of streptomycetes was found to reduced from 2.54×10^8 to 1.14×10^5, 7.2×10^8 to 7.10×10^5, 8.73×10^8 to 6.5×10^5, 7.88×10^8 to 1.0×10^6 and 1.78×10^7 to 4.44×10^4 CFU/g in trials 1, 2, 3, 4 and 5, respectively during the composting period (Figure 4.12). However, in municipal solid waste 61.1% of streptomycetes have been reported (Rebollido et al., 2008). Since, ISP-4 medium is not strictly selective for streptomycetes, therefore only the colonies with aerial mycelium (powdery, wrinkled, or pasty) were counted sensibly as reported by Ryckeboer et al. (2003). The higher range of counts at the end of composting period might be due to good amount of lignin available in the source material. On analyzing the results by ANOVA, actinomycetes and streptomycetes varied significantly among all the trials ($p < 0.05$).

- **Fungi**

Fungus is an important microbe participating in biodegradation of the organic matter present in compost material. It actively participates in lignocellulosic degradation due to its highly efficient enzymatic system, it plays as one of the major contributor of compost stability and maturation (Sánchez, 2009). The fungal count was found to decrease from 5.2×10^7 to 4.20×10^4, 2.13×10^8 to 9.1×10^5, 1.99×10^8 to 6.79×10^5, 1.20×10^8 to 2.1×10^5 and 8.90×10^7 to 1.1×10^5 CFU/g in trials 1, 2, 3, 4 and 5, respectively from 0th to 20th day of composting (Figure 4.13). The major decline in fungal count was observed from 4th till 8th day of composting i.e. in the thermophilic stage.

This decline in number was on account of higher temperature range of 50°C to 56°C. As the highest temperature reached in trial 3 the fungal count decreased by about 1000 folds.

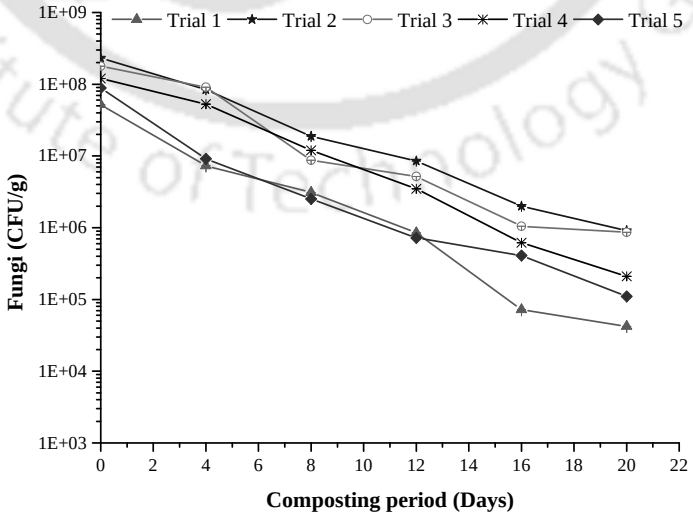


Figure 4.13: Variation of fungi during composting period

It was lower than that of trial 2, this supports the fact that fungi are less resistant to higher temperatures and cannot tolerate temperature above 35-40°C. As highest temperature was observed in trial 3, the major drop in fungal count from 1.99 $\times$ 10^8 to 6.79 $\times$ 10^5 CFU/g was observed in this trial. This was the highest reduction among all other trials. On analyzing the results by ANOVA, a significant variance among all the trials was observed ($p < 0.05$). However, it has also been reported that fungal count had reduced completely in vegetable waste composting (Bhatia et al., 2012). However, in municipal solid waste composting fungal count decreased from 4.5 $\times$ 10^6 CFU/g to 6.3 $\times$ 10^3 CFU/g in third week of composting period (Hassen et al., 2001; Gajalakshmi and Abbasi, 2008). In present study considerable amount of fungal inactivation, around 1000 fold was observed in 20 days. The total inactivation was not observed may be due to large amount of cellulose, hemicellulose and lignin available in the source. Thus, organic matter is still available to be degraded further.

• **Total, fecal coliform and entrococci**

The presence of coliform bacteria is often used as an indicator of overall sanitary quality of water and environments (Hassen et al., 2001). The high temperature helps in destruction of some common pathogens and parasites. Pathogens are also destroyed or controlled by competition with other microbes, antagonistic relationship, antibiotic of inhibiting substances produced by microbes and time of survival (Kalamdhad et al., 2008). The recommended fecal coliform and streptococci densities for compost hygienization are 5.0 $\times$ 10^2 and 5.0 $\times$ 10^3 MPN/g, respectively (Kalamdhad and Kazmi, 2009). The initial and final count of total coliform and fecal coliform were tested for all the trials. The result shows that maximum reduction of total coliform was obtained in trial 3 from 1.1 $\times$ 10^{10} to 2.4 $\times$ 10^2 MPN/g (Table 4.2).

Table 4.2: Total and fecal coliform count on initial day and final day of composting

	Total coliform (MPN/g)		**Fecal coliform (MPN/g)**	
Trial	**Initial day**	**Final day**	**Initial day**	**Final day**
1	1.50×10^{11}	1.50×10^{6}	1.50×10^{10}	9.30×10^{3}
2	2.40×10^{11}	1.50×10^{5}	4.30×10^{7}	4.30×10^{3}
3	1.10×10^{10}	2.40×10^{2}	1.50×10^{6}	1.50×10^{1}
4	2.10×10^{12}	1.50×10^{4}	4.30×10^{8}	1.50×10^{3}
5	1.49×10^{16}	1.49×10^{9}	2.80×10^{9}	1.50×10^{7}

The fecal coliform (FC) values given in (Table 4.2), show that trial 3 had greatest reduction of FC from 1.5 $\times$ 10^6 to 15 MPN/g. Trial 1, 2 and 4 also depicted the decrease

in concentration of fecal coliform after 20 days of composting. But the decrease was not enough to consider the compost to be hygienic. The results indicated that trial 3 yielded most hygienic compost after maturation of primary stabilized composts. Entrococci population was observed very less in all the trials. The count observed till 4th day of composting was in range of 1×10^2 to 2×10^3 CFU/g. Later the population of these microbes was not at all observed. On analyzing the results by ANOVA, a significant variance was observed among all the trials ($p < 0.05$) for total, fecal coliform and entrococci . The development of high degrading temperature in the compost might have abolished the growth of these pathogens making the compost more stable and hygienic for its use in field.

- **Metal analysis of the compost**

The total concentration of metals increases with the increase in organic matter degradation with the release of CO_2 and water (Singh and Kalamdhad, 2013a). Presence of heavy metals in compost makes it too hazardous to be used in field. Enhancement of heavy metals in soil laden with compost can increase the heavy metal concentration in plants. Table 4.3 demonstrates the variation in total concentration of metals (Pb, Zn, Ni, and Cd) during 20 days of composting period. The order of heavy metals in the compost was Pb>Ni>Zn>Cd. Whereas, Zn concentration was reported to be highest of about 220-370 mg/kg in compost of sewage sludge (Chiang et al., 2007). However, all total metals concentration decreased for the compost of municipal solid waste (Castaldi et al., 2006).

Total metal concentration give the information about the level of contamination, however no information is derived regarding their biovailibility and leachability in the environment. Whereas, present study focuses on the scenario of the toxicity of heavy metals in the compost and the microbes present in that contaminated environment.

Table 4.3: Total metals concentration during composting period (mg/kg dry matter)

Days	Pb	Cd	Zn	Ni
0	789 ± 6	42.6 ± 1	122.4 ± 2	251 ± 1.1
4	815.5 ± 4.5	46.3 ± 1.4	132.3 ± 1.4	243 ± 3.1
8	832.5 ± 2.5	48.7 ± 2.3	154.6 ± 2.5	250 ± 3.3
12	786 ± 4	49.2 ± 2.5	181.2 ± 2.1	231 ± 7.3
16	778 ± 7	49.7 ± 3.2	167.3 ± 2.3	242 ± 2.1
20	845.5 ± 4.5	53.2 ± 1.1	181.1 ± 3.1	254 ± 4.2

Note:(mean $\pm$ SD, n=3) SD- standard deviation, (ANOVA, $p < 0.05$)

• **Water soluble heavy metals**

With the decomposition of organic matter, the fraction of heavy metals increases comparatively, but the water soluble fraction of metals may decrease due to the changes of ionic and oxidising conditions of surrounding compost environment. The extent of total metal content in compost is not sufficient to predict its potential toxicity, but the bioavailability and leachability of heavy metals are also important (Singh and Kalamdhad, 2012). It is essential to determine the water soluble fraction of metals as they can contaminate the food chain by being present in the compost (Singh and Kalamdhad, 2013a). Table 4.4 shows the changes in water soluble fractions of Pb, Zn, Ni and Cd during 20 days of composting period. Water soluble concentration of Zn was reported to decrease by 41% during the end of composting period. Water soluble fraction of Ni, Cd and Pb was not detected throughout the composting period. Increase in water soluble concentration of Zn was reported by Singh and Kalamdhad (2012) in water hyacinth composting . A decrease of 95 and 60% water soluble fraction of Pb, Cd and Zn respectively was reported in municipal solid waste composting by (Castaldi et al., 2006).

Table 4.4: Water Soluble metal concentrations (mg/kg dry matter)

Days	**Pb**	**Cd**	**Zn**	**Ni**
0	ND	ND	2.9 ± 0.02	ND
4	ND	ND	3.1 ± 0.01	ND
8	ND	ND	4.0 ± 0.04	ND
12	ND	ND	2.4 ± 0.03	ND
16	ND	ND	2.0 ± 0.01	ND
20	ND	ND	1.7 ± 0.03	ND

Note:(mean $\pm$ SD, n=3) SD- standard deviation, ND-Not detected, (ANOVA, $p < 0.05$)

• **Plant availability of heavy metals (DTPA extractable)**

Diethylene triamine penta-acetic acid (DTPA) extractable heavy metal is the potentially available metal for the plant uptake (Chiang et al., 2007). It is a supplementary approach to find out the potential eco-toxicity of heavy metals (Singh and Kalamdhad, 2012). Table 4.5 shows the variations in DTPA extractable fractions of Pb, Zn, Ni and Cd during 20 days of composting period. DTPA fractions for Pb and Cd were not detectable throughout 20 days. The concentration of Ni was however reported to increase by 10% from initial till the final 20^{th} day. Hydrolysis of metal ions due to alteration in pH contributes to the variability of metals. The concentration of Zn was observed to increase by 80% from its initial concentration. Similarly not detectable Pb and Cd along with increased concentration

of Ni and Zn were reported in water hyacinth composting (Singh and Kalamdhad, 2013a).

Table 4.5: DTPA extractable metal concentrations (mg/kg dry matter)

Days	Pb	Cd	Zn	Ni
0	ND	ND	29.8 ± 0.4	2.0 ± 0.01
4	ND	ND	33.0 ± 0.1	1.6 ± 0.05
8	ND	ND	37.4 ± 2.4	1.4 ± 0.01
12	ND	ND	65.1 ± 0.2	1.6 ± 0.03
16	ND	ND	51.3 ± 0.2	1.7 ± 0.09
20	ND	ND	53.8 ± 0.2	2.2 ± 0.02

Note:(mean ± SD, n=3) SD- standard deviation, ND-Not detected, (ANOVA, $p < 0.05$)

• Leachable heavy metals during composting

Toxicity characterstic leaching procedure (TCLP) depicts the mobility of the organic and inorganic components present in the solid and liquid waste materials (Singh and Kalamdhad, 2013b). Heavy metal found in leachable state can gradually percolate the groundwater table causing contamination. For the compost TCLP should be determined to detect its suitability of being non-hazardous for the land application. Table 4.6 denotes the leachability changes of Pb, Cd, Zn and Ni throughout the 20 days of composting period. Concentration of Pb was reduced by 33% from initial day of composting, however, an increase in Pb concentration was reported in water hyacinth composting by (Singh and Kalamdhad, 2012). Whereas, for Cd the concentration was observed to be reduced by 57%. For Ni, 39% of reduction was reported, whereas 6% of increase was observed for Zn from their initial till the final day concentration. The influence of pH on the solubility of metal ions is well known, either by solubility equilibria or by complexation pH increase affects the solubility of divalent trace metals (Cambier and Charlatchka, 1999).

Table 4.6: (TCLP) Leachable metal concentrations (mg/kg dry matter)

Days	Pb	Cd	Zn	Ni
0	7.5 ± 0.1	2.1 ± 0.02	25.1 ± 0.5	7.8 ± 0.5
4	4.8 ± 0.2	2.3 ± 0.2	25.3 ± 0.4	5.3 ± 0.6
8	8.2 ± 0.6	2.0 ± 0.1	28.8 ± 1.7	4.6 ±2.3
12	4.2 ± 0.4	1.8 ± 0.05	44.9 ± 0.6	5.5 ± 0.5
16	2.2 ± 0.2	1.6 ± 0.07	31.7 ± 1.1	6.6 ± 0.4
20	5 ± 0.4	0.9 ± 0.06	26.7 ± 0.2	4.7 ± 0.3

Note:(mean ± SD, n=3) SD- standard deviation, (ANOVA, $p < 0.05$)

4.1.5 Conclusion

The present study depicted that various microbes played role in water hyacinth composting, temperature and the availability of the substrate decides the survival and degradation activity of these microbes. The combination of feedstock materials plays a key role by varying the organic matter content, thereby dominates the growth patterns of different microbial communities. Highest temperature and degradation was found in trial 3. Bacterial count was highest among all microbes varying from mesophilic to spore forming bacteria according to the temperature. Actinomycetes and streptomycetes count remained higher due to the presence of lignocelluloses in water hyacinth. Fungal count decreased throughout without ceasing. Lower CO_2 evolution rate and OUR in the end signified stability of compost. The VS reduction was not enough, this indicated the degradation of complex lignocellulosic material into monomeric compounds but no complete formation of humic substances was observed. Hence, the compost was stable but yet to be matured. Trial 3 was best among all in terms of stability and microbial dynamics. Lower CO_2 evolution rate and OUR in the end signified the stability of compost.

Higher diversity of microbes resulted in more degraded and stable and pathogen free compost. It was revealed from this study that rotary drum composting of water hyacinth is enriched with numerous microorganisms. Bacteria were high in count playing a substantial role in compost formation. Total heavy metals concentration was in the order of Pb>Ni>Zn>Cd. However, for Pb, Cd and Ni water solubility remained non-detectable, DTPA concentration for Pb and Cd was also found non-detectable. The microbes in water hyacinth compost are metabolically active degrading the organic matter, surviving in the heavy metal loaded compost environment. Finally, it was concluded that isolation and identification of bacteria would be focused on the next phase of the study. These bacterial species can be investigated further for their capability of heavy metal removal in liquids and solid waste sources. Thus, anticipating the application of isolated microbes for the purpose of micro-bioremediation and heavy metal removal by the biosorption process.

4.2 Phase II: Microbial Studies on the Best Trial of Water Hyacinth Compost

4.2.1 Microbial Succession (DNA) from Best Trial

This study focuses on the enumeration and identification of different bacterial communities present in the degradation of water hyacinth. The best combination of waste materials i.e. water hyacinth, cow dung and sawdust in the proportion of 6:3:1 was analyzed using 16S Metagenome sequence method.

• Qualitative and quantitative analysis of gDNA

gDNA was isolated using modified Xcelgen Soil gDNA isolation kit. The quality of gDNA was checked on 0.8% agarose gel (loaded 5 μL) for the single intact band. The gel was run at 110 V for 30 min. 1 μL of each sample was loaded in Nanodrop 8000 for determining A260/280 ratio. The DNA was quantified using Qubit dsDNA HS Assay kit (Life Tech). 1 μL of each sample was used for determining concentration using Qubit® 2.0 Fluorometer.

• Preparation of libraries for 2 X 300 bp run chemistry

The amplicon libraries were prepared using Nextera XT Index Kit (Illumina inc.) as per the 16S Metagenomic Sequencing Library preparation protocol (Part # 15044223 Rev. B). Primers for the amplification of the V3-V4 hyper-variable region of 16S rDNA gene of bacteria and Archaea were designed in Xcelris NGS Bioinformatics Lab (Table 4.7). These primers were synthesized in Xcelris PrimeX facility. The amplicons with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The amplicon libraries were purified by 1X AMpureXP beads and checked on Agilent High Sensitivity (HS) chip on Bioanalyzer 2100 and quantified on fluorometer by Qubit dsDNA HS Assay kit (Life Technologies).

Table 4.7: Primers used in the present study

Sr.No.	Oligo Name	Oligo Sequence (5' to 3')	Primer Length	Product size (Approx.)
1	V3 - Forward	CCTACGGGNGGCWGCAG	17	~ 460 bps
2	V4 - Reverse	GACTACHVGGGTATCTAATCC	21	–

• **Cluster generation and sequencing**

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyser profile, the library was loaded onto Illumina Platform at appropriate concentration (10-20pM) for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions on Illumina Platform. The kit reagents were used in the binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

• **QC on agarose gel**

The library was prepared from compost sample after amplifying the V3-V4 region of the 16*S* gene sequences after the extraction of total DNA from finished compost. The library was in the size range of 600-630 bp. The library was sequenced using the Illumina 2 x 250 bp sequencing chemistry to generate ∼250 Mb of data per sample. The bands on the polyacrylamide gels represent the single-strand-conformation polymorphism (SSCP) patterns. The intensity of bands in the gel represent the reproducibility of DNA extraction method as well as the PCR amplifications (Figure 4.14).

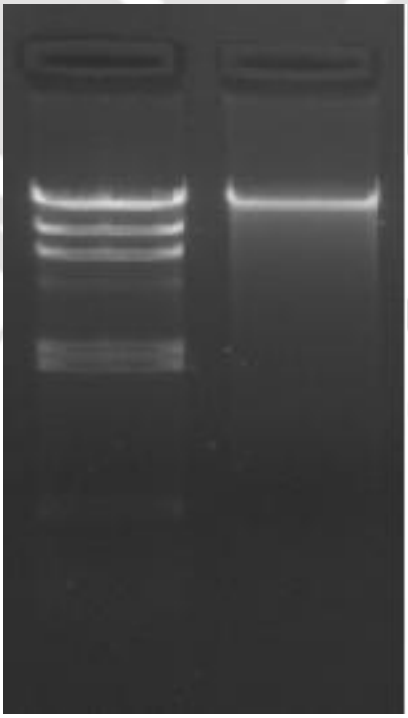


Figure 4.14: QC of gDNA on 0.8% Agarose gel

4.2.1.1 Taxonomic hits distribution

The distribution of taxonomic domains, phyla, and orders for the annotations are represented below in the form of pie charts. Predicted proteins and ribosomal RNA genes in the compost sample are annotated to respective taxonomic level. This information is based on all the annotation source databases used by MG-RAST.

• **Taxonomic hits distribution at phylum level:** Taxonomic hit distribution at phylum level shows that compost has 39.08% *Bacteroidetes*, 36.77% *Proteobacteria*, 10% *Verrucomicrobia* etc. as represented in (Figure 4.15). The *Bacteroidetes* and *Proteobacteria* are the most abundant bacterial phyla among the compost recipe, soil, sediments, and rumen. Cow dung is the source of proliferation of these microorganisms in compost (Yamamoto et al., 2011; Neher et al., 2013). Most members of *Bacteroidetes* and *Proteobacteria* phyla are facultatively or obligately anaerobic and heterotrophic. The phylum *Bacteroidetes* are composed of three large classes of gram-negative, non-spore forming, anaerobic or aerobic and rod-shaped bacteria which play a crucial role during the initial and final stages of composting. It has been reported that *Bacteroidetes* are abundant following the thermophilic stage and play a major role in compost maturation by degrading complex polymers (Franke-Whittle et al., 2005; Yu et al., 2007; Neher et al., 2013)

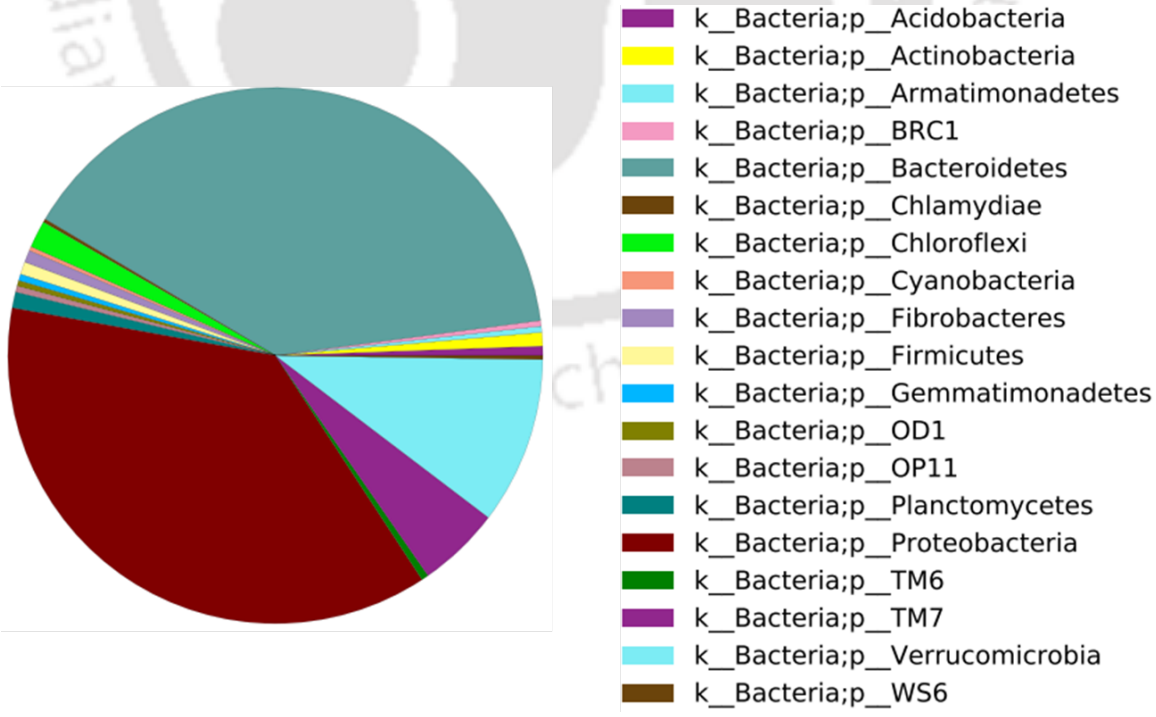


Figure 4.15: Taxonomic hits distribution at phylum level of compost sample

• **Taxonomic hits distribution at class level:** Taxonomic hit distribution at class level shows that compost has 24.21% *Flavobacteria*, 17.57% *Gammaproteobacteria*, 12.22% *Alphaproteobacteria*, 6.78% *Sphingobacteria* etc. as represented in (Figure 4.16). The *Flavobacteria* and *Sphingobacteria* belong to *Bacteroidetes* species, it is known for degradation of starch, cellulose, proteins, chitin and the phenolic and chlorinated compounds (Green et al., 2004; Danon et al., 2008). The higher temperatures of natural compost are a consequence of degradation by *Flavobacteria* during composting process (Liu et al., 2011). Bacteria belonging to *Alphaproteobacteria* have been reported during curing as well as in the noncured compost (Danon et al., 2008). The *Bacillus* sp. was dominant species during the middle and later stages of composting.

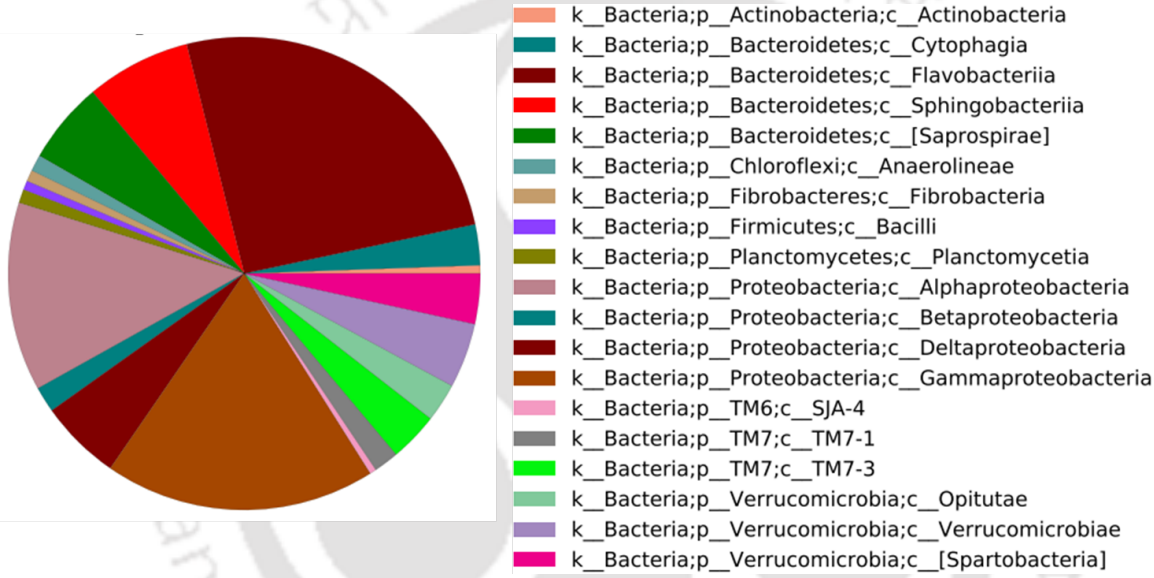


Figure 4.16: Taxonomic hits distribution at class level of compost sample

• **Taxonomic hits distribution at order level:** Taxonomic hit distribution at order level shows that compost has 24.21% *Flavobacteriales*, 7.7% *Alteromonadales*, 6.78% *Sphingobacteriales*, 5.81% *Rhizobiales* etc. as represented in (Figure 4.17). *Flavobacteriales* and *Sphingobacteriales* belong to phylum *Bacteroidetes*, *Alteromonadales* are an order of *Proteobacteria*. *Rhizobiales* are an order of *Alphaproteobacteria* and are considered to be gram-negative. All the bacterial communities belonging to *Bacteroidetes* and *Proteobacteria* are known for degrading ligno-cellulosic compost material, being present during curing of compost as well as in noncured compost (Yu et al., 2007; Franke-Whittle et al., 2009). The rhizobia are known to fix nitrogen by the symbiotic relation with plant roots. *Rhizobiales* play an important role in the denitrification of manure compost pellets and emitting N_2O (Yamane, 2013).

From the above study it can be concluded that Amplicon sequencing and analysis has

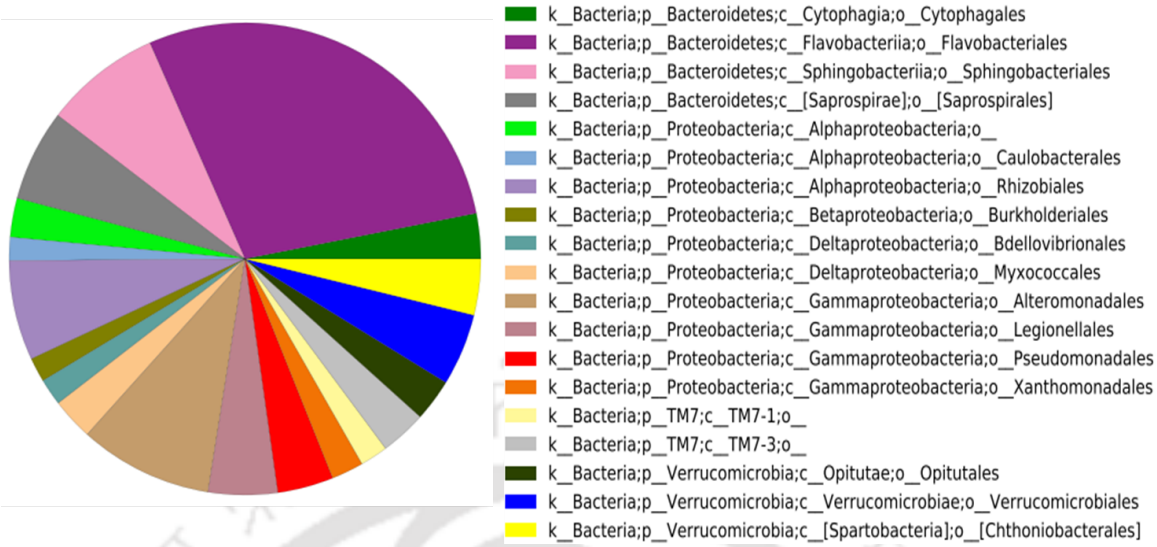


Figure 4.17: Taxonomic hits distribution at order level of compost sample

been carried out for compost sample with following details. At phylum level, compost sample is enriched with *Bacteroidetes*, at class level compost sample are enriched with *Flavobacteria*. At order level, compost sample is enriched with *Flavobacteriales*. Bacteria are the most abundant microorganism in the compost.

4.2.2 Isolation and Identification of Bacteria During Rotary Drum Composting of Water Hyacinth

Water hyacinth (*Eichhornia crassipes*) is the fast growing and robust plants on earth. Composting followed by its use in land application has come out to be the best method for treatment and utilisation of this weed (Malik, 2007). However, huge amount of heavy metals have been reported in compost of water hyacinth (Singh and Kalamdhad, 2012). Additionally, a high count of actinomycetes, streptomycetes, bacteria and fungi was also observed throughout whole period of rotary drum composting of water hyacinth. Bacteria are diversified in different ecological habitats. They have valuable uses in environmental microbiology. Numerous microbes have been isolated from different waste sources for their exploitation in varied microbe-based technologies. Contaminated regions such as industrial waste, wastewater, soil, municipal waste and sewage sludge have been used as source for the isolation of the most robust microbes (Pal and Paul, 2004; Congeevaram et al., 2007; Bahig et al., 2008; Jiang et al., 2008; Shoeb et al., 2009; Çolak et al., 2011; Bautista-Hernández et al., 2012) .

Composting is a rigorous process of aerobic degradation of organic waste by the metabolic activities of the microorganisms, therefore it serves for the abundancy of metabolically ac-

tive microbes (Hassen et al., 2001; Ryckeboer et al., 2003; Anastasi et al., 2005; Bhatia et al., 2013; Pan and Sen, 2013). The microbes are dependent on the surrounding conditions such as the temperature, availability of organic waste, pH, species diversity and availability of heavy metals. Large variety of mesohphilic, thermotolerant microorganisms have been reported in composting of different types of waste materials, it includes bacteria, actinomycetes, streptomycetes and fungi (Hassen et al., 2001; Ryckeboer et al., 2003). In the previous study of the microbial dynamics of the water hyacinth compost, substantial bacterial communities had been reported . Thus, the water hyacinth compost is a potential source of microbes.

For the current phase work the water hyacinth compost has been utilised as a source for the isolation of the most robust and persistent microbes. However no work has been reported regarding detection, isolation, and identification of bacteria during rotary drum composting of water hyacinth. Hypotheses is that the bacterial community capable for sustaining the variable conditions of composting from the initial till the final day could be further utilised for the purpose of micro-bioremediation. Hence, there is an immense possibility in using them for bioadsorption, biotransformation and/or bioaccumulation the heavy metals (Anastasi et al., 2005; del Carmen Vargas-García et al., 2012). Accordingly, in the present study basic parameters to check compost quality and stability were performed, along with this culture dependent and culture independent approaches were adopted to isolate and identify the bacterial strains.

- **Isolation and identification of microbes**

Media optimization was done on the basis of their maximum consistency and ability to support growth of bacterial populations. For this, the different selected media were used for culturing bacterial populations during the whole composting period. From the previous study it was found that about 10^4-10^7 dilutions were consistently giving a good colony count in case of mesophilic bacteria. The initial count was as high as 50 for glucose minimal media which decreased to 18 gradually. This was synchronous with nutrient rich media which yielded 41 colonies initially and got reduced to 17 colonies in the end. The total number of colonies obtained from the compost was 656. The glucose minimal media gave the maximum 222 colonies followed by nutrient media with 214 colonies. Sucrose minimal media and tryptic soy agar media yielded a total of 102 and 118 colonies, respectively. The quadrant streaking was continued for 10 cycles which concluded with total 12 isolates from all four media. A total of 12 distinguishable isolates were obtained at the end. All the discrete isolates were undertaken for morphological and biochemical tests as described in the Table 4.8. Gram staining and phase contrast microscopy gave the shape identification

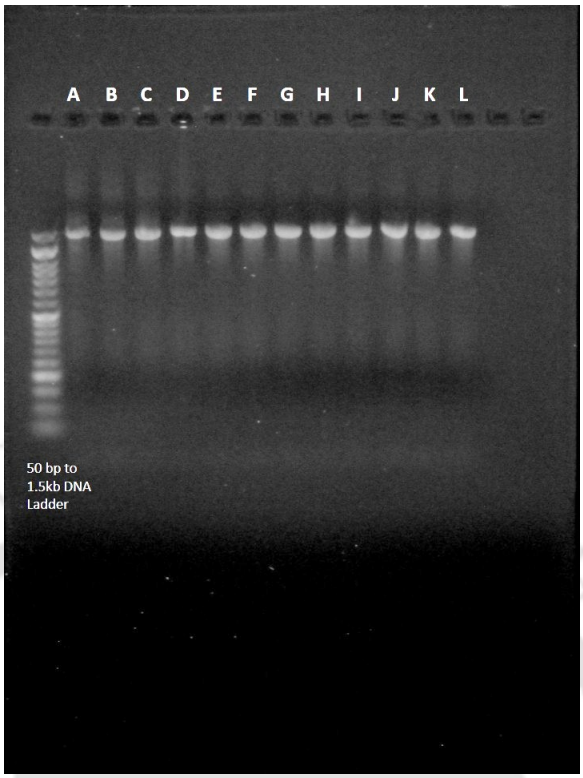


Figure 4.18: Agarose gel image of PCR samples

of bacterial colonies, which were spotted to be in different forms such as, rod, circular and oval shapes (Bhatia et al., 2013). The colours of the strains were mostly, white, yellow, cream, orange and pink. DNA was extracted from the culture pellets using GeNei DNA Purification kit, PCR was performed with primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (forward) and 1492R(5'-CGGTTACCTTGTTACGACTT-3') (reverse) primers. PCR samples were tested by agarose gel electrophoresis (Fig. 4.12) and send to Xcelris Labs Ltd. for 16S rRNA gene sequencing, thereafter the gene sequence was submitted to NCBI Gen-bank database

• **Molecular identification**

Sequence analysis of the 16S rRNA gene is a fast and accurate method to identify the phylogenetic position of bacteria. Full-length (about 1500 bp) 16S rDNA of all 12 bacterial isolates was sequenced and used to construct a phylogenetic tree. The 16S rDNA gene sequence was used to carry out BLAST alignment of NCBI genebank database. Based on maximum identity score, sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5 (Tamura et al., 2013). Figure 4.19 shows

Table 4.8: Biochemical characterization of bacterial isolates

Tests	*Bacillus subtilis* AK4	*Bacillus* sp. AK8	*Bacillus thuringiensis* AK7	*Bacillus cereus* AK11	*Bacillus* sp. AK10	*Bacillus cereus* AK9	*Bacillus badius* AK	*Enterobacter* sp. AK1	*Bacillus cereus* AK6	*Enterobacter* sp. AK2	*Enterobacter* sp. AK3	*Enterobacter* sp. AK5
Cell shape	Rod	Rod	Rod	Rod	Rod	Oval	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	+	+	+	+	-	+	+	-	+	-	-	-
Spore stain	+	+	+	+	+	+	+	-	+	-	-	-
Capsule	-	-	-	-	-	+	+	-	+	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	-	+	+
Oxidase	+	+	+	+	+	+	-	-	-	-	-	-
Methyl Red	-	-	-	+	+	+	-	-	+	+	-	-
Voges-Proskaur	+	+	+	+	+	+	+	+	+	-	+	+
Motility	+	-	+	+	+	+	+	+	+	+	+	+
Oxidation	+	+	+	+	+	+	+	-	+	-	-	-
Fermentation	+	+	+	+	+	+	+	+	+	-	+	-

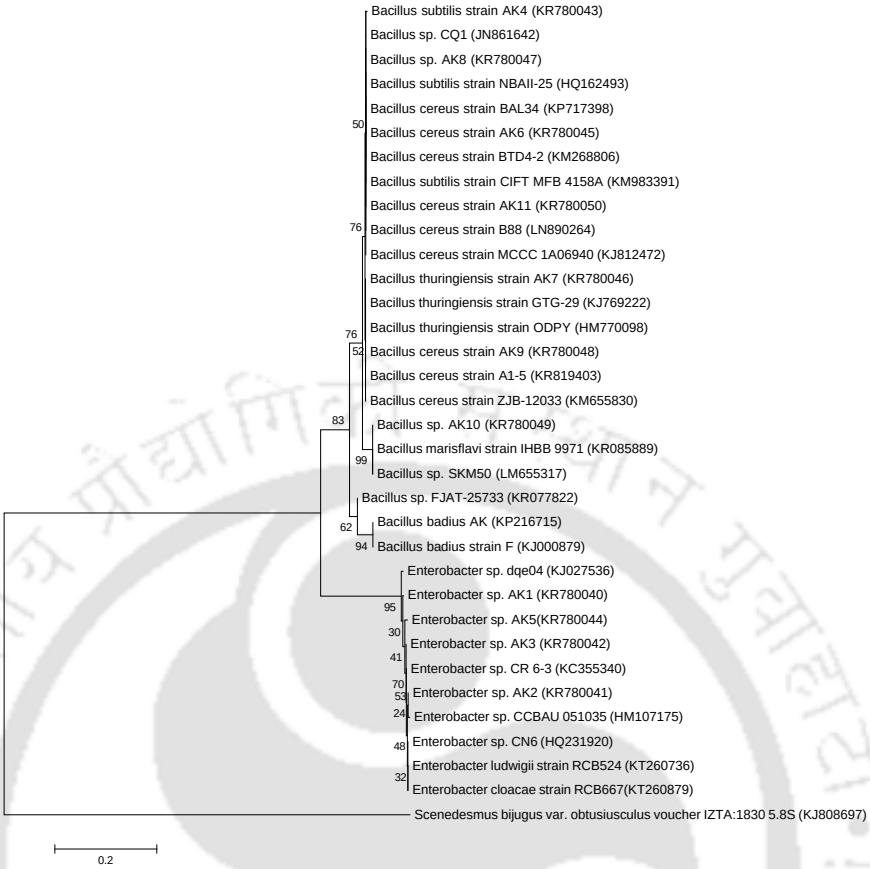


Figure 4.19: Phylogenetic Tree of bacterial isolates

the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2- parameter method and are in the units of the number of base substitutions per site (Kimura, 1980). The accession nos. received from NCBI were KR780040-KR780050 and KP216715. The bacterial groups were *Enterobacter*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus badius*, *Bacillus thuringiensis* (Figure 4.19). Although composting is a aerobic process members of facultative anaerobic family i.e. *Bacillus* are much prevalent which signifies the versatility and survival capacity of these microbes.

4.3 Conclusion

Amplicon sequencing and analysis has been carried out for compost sample with following details. At phylum level, compost sample is enriched with *Bacteroidetes*, at class level compost sample comprises of *Flavobacteria*. At order level, compost sample is enriched with *Flavobacteriales*. Rotary drum composting of water hyacinth is enriched with numerous microorganisms. Bacteria are the most abundant microorganisms in the compost, which degrade the organic matter by surviving in the heavy metal loaded compost environment. Twelve new robust bacterial strains were isolated as being the most consistent throughout the 20 days of composting period. Culture dependent and culture independent approach gave a spectacle of new bacterial strains. The microbes not only survived the rigorous variations of composting process, but also the presence of the toxic heavy metals of the compost. Reduction in water soluble, leachability and DTPA values of heavy metals indicated the efficient degradation of the organic matter by the microbial activities during the composting process. These bacterial species can be investigated further for their capability of heavy metal removal in liquids and solid waste sources. In this way these bacteria can contribute for the purpose of micro-bioremediation.

Metabolically active bacteria leads to degradation of the organic matter, also they are surviving in the heavy metal loaded environment of water hyacinth compost formation. Twelve new robust bacterial strains were isolated as being the most consistent throughout the 20 days of the composting period. Huge degradation of organic matter, temperature, pH and heavy metals was observed. Both the culture dependent and culture independent approach gave a spectacle of new bacterial strains. They not only survived the rigorous variations of composting process, but also the presence of the toxic heavy metals of the compost.

"It isn't pollution that's harming the environment. It's the impurities in our air and water that are doing it."

Dan Quayle

5

Biosorption Studies of Pb(II) and Cd(II) With Live and Dried Biomass of *Bacillus badius* AK

This chapter aims the performance of the isolated bacterial strain *Bacillus badius* AK in removal or biosorption of heavy metals such as Pb(II) and Cd(II). In addition, the live and dead form of bacterial biomass was tested for the Pb(II) removal and dead form of Bacterial biomass was tested for Cd(II) removal. The experiments were conducted in batch system. The optimum parameters required for the removal of Pb(II) and Cd(II) have been discussed in detail.

5.1 Biosorption of Pb(II) by Live Cells (Non-pretreated) of *Bacillus badius* AK Strain Isolated From Water Hyacinth Compost

Mercury, lead and cadmium called "the big three" are in the limelight because of their impact on the environment. Among all the toxic heavy metals, lead is potentially threatening for human health and animals, affecting children, pregnant women and adults, risking their lives with problems of delayed growth, anemia, kidney diseases, cardiovascular dysfunction and reproductive problems (USPEA, 2011). The World Health Organization (WHO) and Bureau of Indian Standards (BIS) recommend threshold limit of 10 μg/L for lead in the drinking water quality guidelines (Singh et al., 2012). Microorganisms have a high surface area-to-volume ratio because of their small size, thus provides a large contact area that can interact with metals in the surrounding environment. Different kinds of the intrinsic or induced mechanisms can be involved in the accumulation of metals by microorganisms e.g. adsorption, precipitation, complexation, extracellular polymers and active transport into the cell (Ledin, 2000; Gadd, 2009). Biosorption of heavy metals using bacterial, fungal and algal biomass (living or dead cells) has been found as a potential

alternative to the conventional techniques, since it does not produces secondary sludge, has higher selectivity, more efficient, easy to operate and cost effective for treating large volumes of water and wastewater. Microbes thriving in metal polluted environment adapt mechanism of survival by developing resistance to the metals.

For the purpose of biosorbent, different robust microbes have been isolated from various waste sources such as, wastewater, soil, sewage sludge, industrial effluent, and compost of horticulture and municipal wastes. Different species of *Pseudomonas*, *Bacillus*, *Microbacterium*, *Micrococcus*, *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Atrhobacter* are reported to reduce Pb, Cr, Zn, Cd, Cu, Ni and Cd (Belimov et al., 2005; Congeevaram et al., 2007; Mishra and Doble, 2008; Choudhary and Sar, 2009). A little work has been reported on use of bacterial biosorbent in live strain form, however, live form of bacteria has been considered with its optical density, but bacterial colony forming units in context with biosorption has never been reported. Also the growth kinetics of bacteria in relation to the effects of heavy metals is lacking.

In present study, the water hyacinth compost was used as a source for isolation of *Bacillus badius* AK, it has been used to evaluate its metal (Pb(II)) biosorptive behaviour in live form. High microbial count of robust bacteria, actinomycetes, streptomycetes and fungi had been reported in rotary drum compost of water hyacinth. Studies are reported on reduction in bioavailability and leachability of heavy metals during rotary drum composting of water hyacinth (*Eichhornia crassipes*) by Singh and Kalamdhad (2013a). The potential use of *Bacillus badius* AK as biosorbent for removal of Pb(II) was studied in detail. Attempts were made to standardize the Pb(II) removal with the effects of different parameters such as temperature, pH, contact time, biomass, metal concentration, and rotational speed on bacteria mediated biosorption.

5.1.1 Batch Biosorption Studies

• Pb and glucose minimal media compatibility study

The media was essentially needed to keep the metal in solution and not let it precipitate, for the microbes to interact with the metal. As the anthropogenic activities change the pH of the surrounding environment, the metals present in vicinity would also be affected (Volesky, 2007; Fomina and Gadd, 2014). Figure 5.1 shows that after pH 5 there was gradual reduction of soluble Pb(II), after pH 5.5 there was complete precipitation, it was required to maintain the pH below pH 5 for lead to remain soluble. Significant differences were observed between all the triplicates ($p < 0.05$). Similar study was reported for Pb(II) removal with *Bacillus* sp. by Çolak et al. (2011).

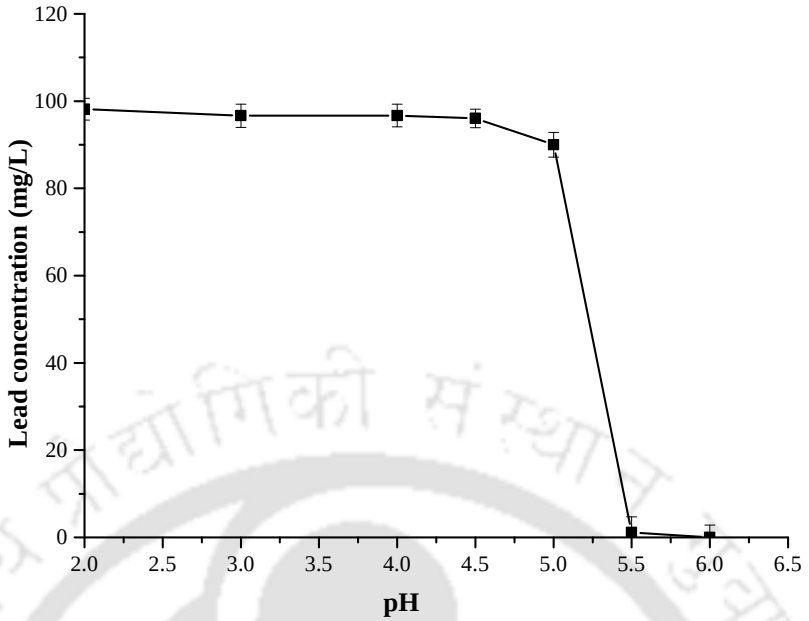


Figure 5.1: Compatibility of Pb(II) with glucose minimal media at different pH values

• **Effect of pH**

pH is one of the most influential factors when it comes to biosorption by bacterial adsorbent. The condition of surrounding pH decides the availability of active sites on cell wall. Figure 5.2 indicates that pH had a significant effect on biosorption of Pb(II) by *Bacillus badius* AK, pH 4-4.5 gave the optimum condition for biosorption by the bacterial strain, the live strains were active in removing Pb(II) with optimum value of 1.1×10^{11} CFU/mL. On analyzing the results by ANOVA, a significant variance was observed among all the trials ($p < 0.05$). The optimum pH for Pb(II) removal by *Bacillus thuringiensis* was reported as 6 (Oves et al., 2013), the amount of Pb(II) uptaken by *B. pumilus* and *B. cereus* increased with the increase in pH from 1 to 6 (Çolak et al., 2011).

As stated by Bueno et al. (2008), if pH increased above the isoelectric point of biomass, more negatively charged ligands such as carboxyl and phosphate groups are available, subsequently increasing the attraction for positively charged metal ions. In present study, effect of lower pH could be explained as the competition between the metal cations and protons to bind the active sites on cell wall, thereby lowering the metal uptake. However, decrease in biosorption at higher pH value might be attributed to the available species of Pb(II), such as formation of $Pb(OH)_2$ compound in alkaline conditions. Similar studies were carried out, where Zn uptake increased within the pH range of 3 to 5 for live cells of *Streptomyces ciscaucasicus* strain (Li et al., 2010), Cd removal by live cells of *Sphingomonas paucimobilis* was reported to be best at pH 6 (Tangaromsuk et al., 2002).

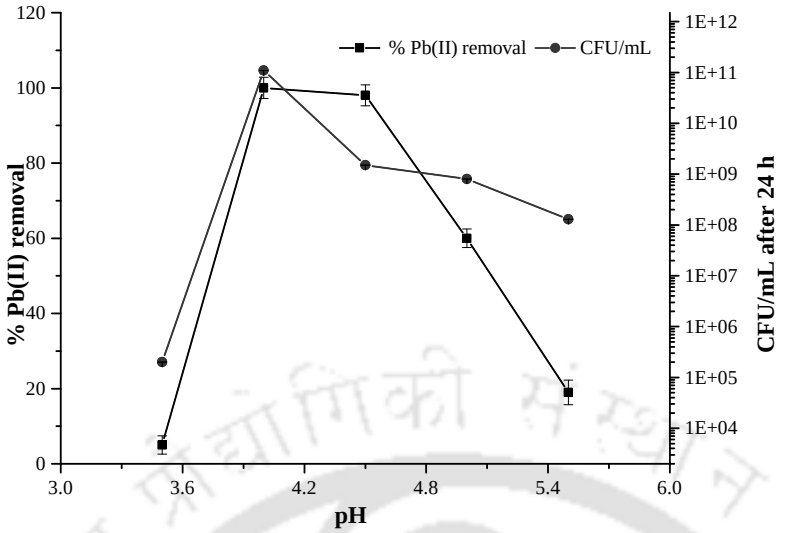


Figure 5.2: Batch Biosorption studies for Pb(II) removal: Effect of pH

- **Effect of temperature**

Microbial metabolism and population dynamics are dependent on the temperature variation (Liang et al., 2003). Temperature affects the configuration and stability of cell wall, causing ionization of chemical moieties, and the binding sites of bacterial surface (Congeevaram et al., 2007). For the present study, bacterial strain was mesophilic, consequently temperature range of 20-40°C was optimized. On analyzing the results by ANOVA, a significant variance was observed among all the trials ($p < 0.05$). Thus, different temperatures

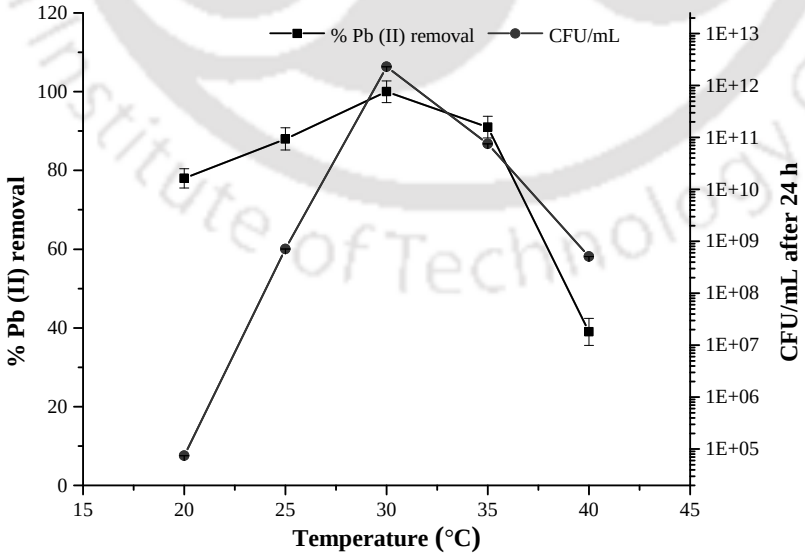


Figure 5.3: Batch Biosorption studies for Pb(II) removal: Effect of temperature

were selected such as 20, 25, 30, 35 and 40°C. Apart from bacterial growth, the temperature affects the biosorbing capacity also, increase in Cu adsorption had been reported with the increase in temperature above 35°C (Xinjiao, 2006). Figure 5.3 shows the effect of temperature on biosorption, it indicates that 30-32°C was optimum temperature, with maximum value of 2.3×10^{12} CFU/mL within this temperature range.

• Effect of rotational speed

For a biosorption system, agitation is required to maintain proper interaction between the active sites on cell wall and metal ions. Homogenous mixing of media and bacterial culture was achieved by horizontal shaking of the flask. Xinjiao (2006) stated that rotational speed affected the capacity of *Cladosporium* sp. in absorbing Cu^{2+} ions from the aqueous solution. Thus, optimization of rotational speed was essential. The investigation of highest growth and removal of Pb(II) at different rotational speeds from 80 to 180 rpm is represented in Figure 5.4. It suggests that rotational speed range of 150-180 rpm was best for the biosorption study, in consideration with the microbial growth of 1.6×10^{12} CFU/mL in correspondence with maximum Pb(II) removal. Significant differences were observed between all the triplicates ($p < 0.05$). Reduction of biosorption beyond 180 rpm would be attributed towards the collision of microbes at higher agitation speed.

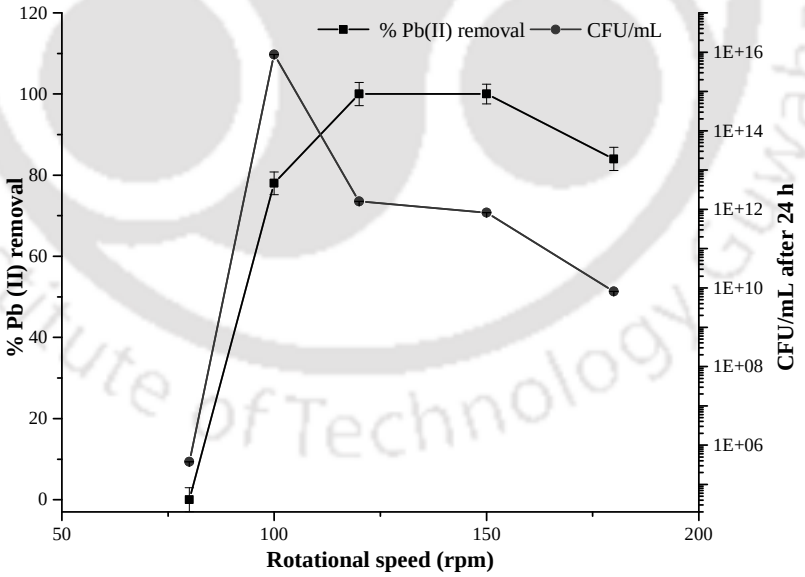


Figure 5.4: Batch Biosorption studies for Pb(II) removal: Effect of rotational speed

• **Effect of initial Lead concentration**

Figure 5.5 depicts that maximum biosorption was recovered in the range of 100-150 mg/L of Pb(II). The decrease in biosorption capacity at higher metal concentration may be attributed towards the unavailability of adsorption sites on bacterial cell wall with respect to the metal ion concentration. As Bueno et al. (2008) and Oves et al. (2013) stated that at lower metal concentration, biosorption percentage is likely to be higher because all metal ions could interact with the binding sites. Microbes showed the optimum growth of 1.3 $\times$ 10^{12} CFU/mL within 100-150 mg/L range of Pb(II), along with maximum metal removal. Beyond this range, microbes lost their viability and action. ANOVA analysis with ($p < 0.05$) depicted significant differences among all the triplicates.

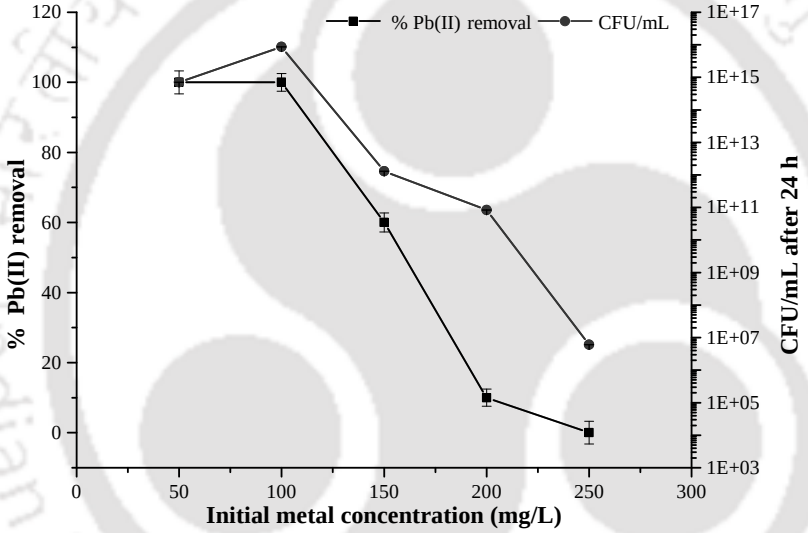


Figure 5.5: Batch Biosorption studies for Pb(II) removal: Effect of initial Lead concentration (mg/L)

• **Effect of initial biomass**

To examine the effect of the initial biomass concentration, the experiments were performed at different biomass culture such as 5, 10, 15, 20 and 30 mL at 100 mg/L of Pb(II) concentration. The test was carried out at optimum temperature of 30°C and pH 4, by incubating for 24 h at 150 rpm. Figure 5.6 shows the results after 24 h, which suggests that 20 mL could efficiently remove 100 mg/L of Pb(II) from the solution, along with the maximum amount of bacterial growth (9.1 $\times$ 10^{14} CFU/mL). Thus, 20 mL was an optimum dosage for 100 mg/L of Pb(II). Significant differences were observed between all the triplicates ($p < 0.05$). Different biomass concentrations such as, 0.05, 0.1 or 0.25 g (dry weight)

of bacterium CCNWRS33-2 have been reported to play role in biosorption capacity of the bacteria (Wei et al., 2009).

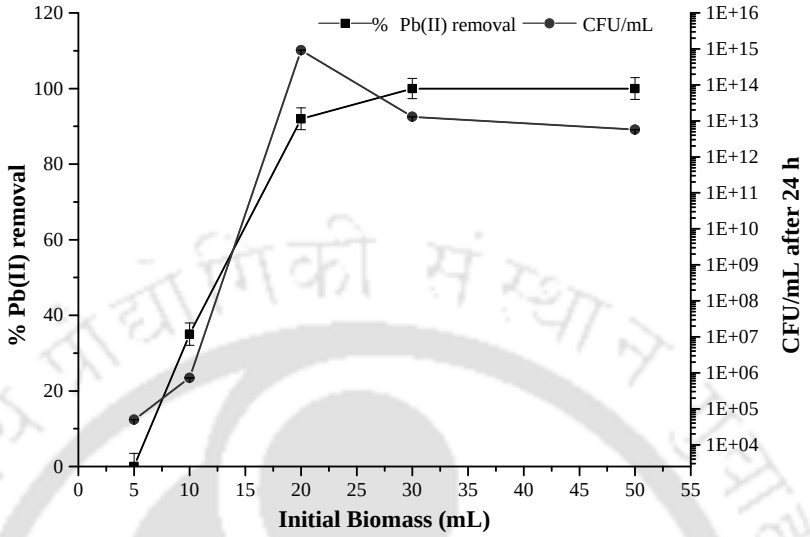
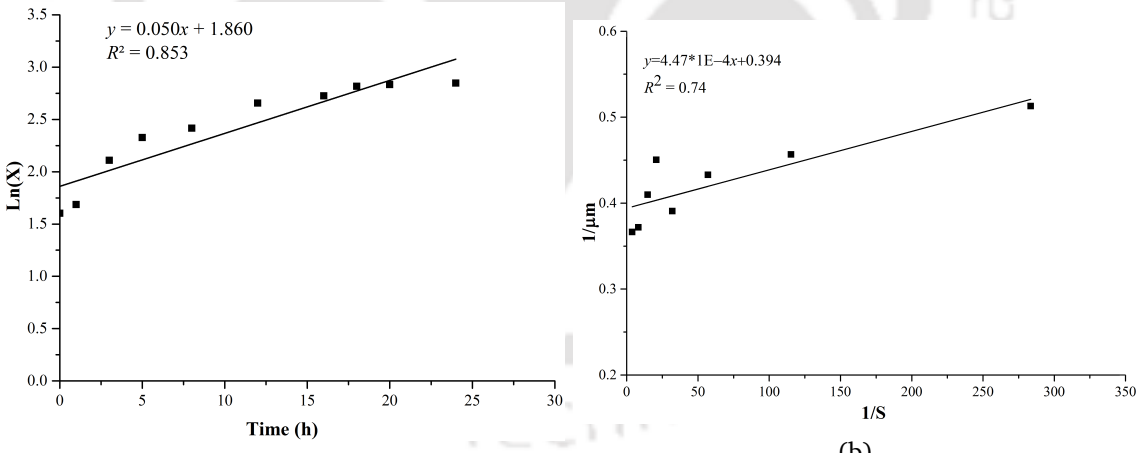


Figure 5.6: Batch Biosorption studies for Pb removal: Effect of initial biomass (mL)

- **Effect of heavy metal on bacterial growth**



(a) (b)

Figure 5.7: (a) Specific growth rate (μ) of *Bacillus badius* AK (b) Maximum specific growth rate (μm) of *Bacillus badius* AK

Microbial growth in presence of the environmental contaminant mostly results into biodegradation of the contaminants. The metabolic activities and substrate utilization of microbes can cause removal of contaminating pollutants (Okpokwasili and Nweke, 2006). Monod's model of microbial growth has been fitted in the present study. Glucose was added

in the minimal media as the substrate for microbial growth. Effect of Pb(II) on bacterial growth kinetics was estimated. 100 mg/L of Pb(II) was present in media as a contaminant.

The specific growth rate (μ) of *Bacillus badius* AK was estimated in batch culture loaded with Pb(II) (100 mg/L). Figure (5.7a) depicts μ of *Bacillus badius* AK as 0.05/h. Maximum specific growth rate (μm) was reported to be 2.54/h (Figure 5.7b).

• Biosorption kinetics

In order to determine the effectiveness of a biosorption system, it is essential to predict it via kinetic models. In this study, Lagregen's pseudo first order (Eq. 3.8) and second order kinetics model (Eq. 3.9) were used to express the experimental data. The linear forms of the first and second order kinetics were calculated, and are presented in the Table 5.1. First order rate constant and q_e values were calculated using least square method. The amount of metal uptake by bacteria at equilibrium (q_e) was calculated for the first order and second order model using equation (Eq. 3.8) and (Eq. 3.9). In order to select the best fit kinetic model, Chi-square (χ^2) and Root mean square error (RSME) test were done (Eq. 3.10 and Eq. 3.11). Where q_t and q_m (meq/g) are metal sorption capacity at time t calculated using experimental data and model, respectively. Where q_{ti} and q_{mi} (meq/g) are metal sorption capacity at time t calculated using experimental data and model, respectively.

Table 5.1 shows comparison of results of the two models and both indicate that pseudo-second order model fits better with R^2=0.987, than pseudo- first order. Thus, from kinetics study it was observed that equilibrium was reached after 6 h. Also, both kinetic models seem to fit, which was also suggested by Harvey and Leckie (1984). Similar results were also reported using *R opacus*, *Bacillus* sp., *Streptomyces ciscaucasicus* (Tunali et al., 2006; Bueno et al., 2008; Cayllahua et al., 2009; Li et al., 2010; Çolak et al., 2011) to the biosorption of metallic ions. This indicated that the kinetic complexities of Pb(II) biosorption onto bacteria and species-varation in surface structure may result in adsorption behaviour that was substantially more complex than that reported for purely inorganic systems.

Table 5.1: Comparison of pseudo-first order and pseudo second order models

Initial conc. of Pb (meq/L)	q_e	**Pseudo first order kinetics**					**Pseudo second order kinetics**				
		R^2	k_1 (L/h)	q_{eq} (meq/g)	χ^2	RMSE	R^2	k_2 (L/h)	q_{eq} (meq/g)	χ^2	RMSE
0.965	0.165	0.966	0.288	0.185	0.006	0.0149	0.987	0.288	0.205	0.0057	0.0139

• **Isotherms study**

Adsorption isotherms estimate the concentration of sorbate as a function of its concentration in the soluble form. This graphical representation gives valuable information about the bisosorbent's applicability. In present study, commonly used isotherm models viz. Langmuir and Freundlich isotherms were adopted to predict the behavior of metal uptake by *Bacillus badius* AK (Li et al., 2010; Çolak et al., 2011). In other studies it had been observed that regression coefficients obtained from Langmuir and Freundlich models were significant (Oves et al., 2013). The weight of biomass considered for calculation was initial biomass, which was 12.5 mg dry weight. It can be seen in Table 5.2 that Langmuir model f itted better than Freundlich, with value of R^2=0.97. Biosorption results with *Bacillus* sp., *Streptomyces ciscaucasicus*, *Bacillus thuringiensis*, *R opacus* were similarly found to follow Langmuir model (Tunali et al., 2006; Bueno et al., 2008; Li et al., 2010; Çolak et al., 2011; Oves et al., 2013). The predominant phenomenon by which this gram negative rod shaped bacteria was able to interact with Pb(II) was monolayer biosorption.

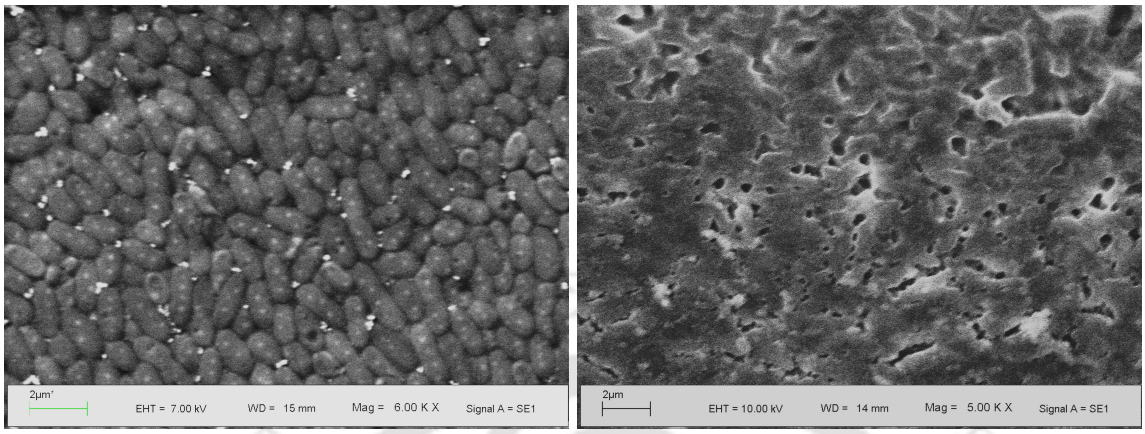
Table 5.2: Comparison of Langmuir and Freundlich isotherm models

Initial concentration of lead (meq/L)	Langmuir isotherm			Freundlich isotherm		
	q_m (meq/g)	b (L/meq)	R^2	K_F	n	R^2
0.965	0.0188	15.87	0.971	1.503	0.675	0.931

• **SEM and EDX analysis**

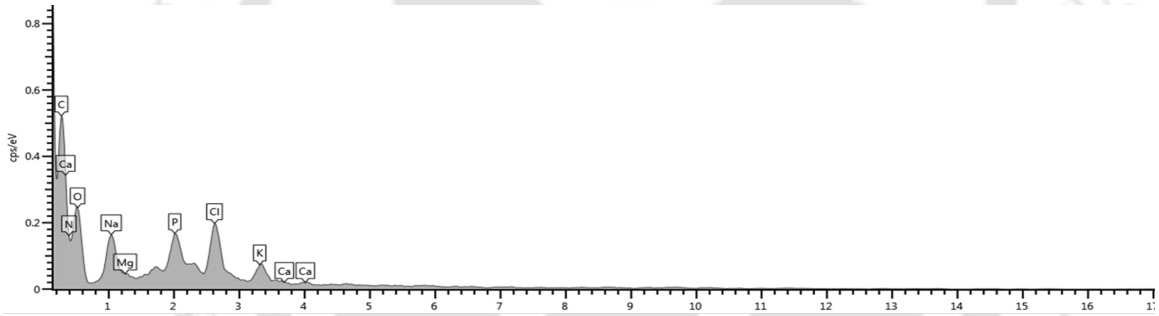
Scanning electron micrograph (SEM) is an important technology to examine the morphological and topological characteristics of a sample. Whereas Energy dispersive X-ray spectroscopy (EDX) gives an explanation about the chemical and elemental characteristics of an adsorbent. In this study, SEM micrographs and EDX spectra of *Bacillus badius* AK were taken before and after the Pb(II) sorption, as represented in Figure 5.8a and Figure 5.8b respectively. SEM micrograph indicated that *Bacillus badius* AK was rod and regular in shape before interacting with Pb(II), after Pb(II) removal, its surface became rough and porous because of Pb(II) adsorption. This observation was confirmed by the EDX analysis, the peaks between 2 and 3 KeV (Figure 5.9a), corresponding to Cl^-, disappeared after Pb(II) adsorption, significant appearance of peaks for Pb(II) were observed within 2 to 3

KeV range (Figure 5.9b). These observations indicated that prominent exchange of ions had also taken during the biosorption process.

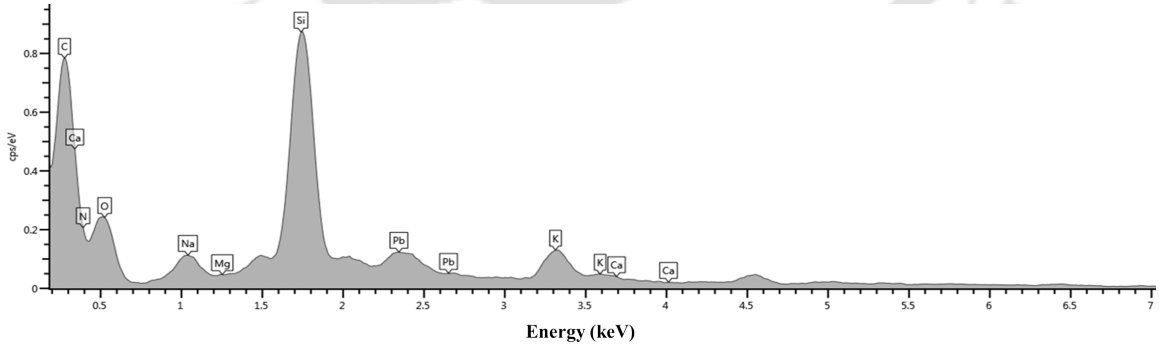


(a) (b)

Figure 5.8: (a) SEM micrograph of *Bacillus badius* AK before Pb(II) biosorption (b) SEM micrograph of *Bacillus badius* AK bacteria after Pb(II) biosorption



(a)



(b)

Figure 5.9: (a) Typical EDX spectra of *Bacillus badius* AK before Pb(II) biosorption (b) Typical EDX spectra of *Bacillus badius* AK after Pb(II) biosorption

The usage of live form of *Bacillus badius* AK isolated from water hyacinth compost was

found to be an easily cultivable and efficient biosorbent for removal of Pb(II) in the aquatic environment. Without any pretreatment and modification of bacterial biomass, the Pb(II) removal was accomplished in live form of bacteria. The biosorption of Pb(II) by *Bacillus badius* AK was affected by pH, initial concentration, rotational speed, temperature and biomass concentration. The maximum biosorption of Pb(II) (100-150 mg/L) occurred with 20mL of biomass having 1.7×10^{16} CFU/mL value. Considering the present finding, dead form of bacteria can also be investigated for Pb(II) and other metals removal.

5.1.2 Conclusion

In the present study, the bacterial strain *Bacillus badius* AK isolated from water hyacinth compost was found to be an easily cultivable and efficient biosorbent for removal of Pb(II) in the aquatic environment. The bacterial biomass was used in its native and non-pretreated state, unlike the dried, freeze-dried or chemically treated biomass. The biosorption of Pb(II) by *Bacillus badius* AK in batch mode was affected by pH, initial concentration, rotational speed, temperature, and biomass concentration. The maximum biosorption of Pb(II) (100-150 mg/L) occurred with 20 mL of biomass having 1.7×10^{16} CFU/mL value. Specific growth rate and maximum specific growth rate of *Bacillus badius* AK was observed as 0.05/h and 2.54/h respectively. The results indicated that bacterial biomass was efficient, robust and cheaper biosorbent for removal of Pb(II). Considering the present finding, dried or pretreated form of bacteria was investigated for Pb(II) and Cd(II) in next phases of the study.

5.2 PHASE III: Biosorption of Pb(II) by Dried Biomass of *Bacillus badius* AK Strain Isolated From Water Hyacinth Compost

There are several physio-chemical techniques applied to remove heavy metals from aqueous solutions which include precipitation, ion exchange, evaporation, electroplating and membrane process. However, these methods have been found uneconomical and inefficient at low metal concentrations, followed by generation of secondary wastes (Kuyucak and Volesky, 1988). Biosorption of heavy metals using bacterial, fungal and algal biomass (living or dead cells) and agricultural waste biomass has been found to be a potential alternative to conventional techniques. Their usage is advantageous on grounds of no secondary sludges production, higher selectivity, easy to operate and economically sound for handling huge volumes wastewater(Sheng et al., 2004; Gong et al., 2005; Tunali et al., 2006; Amarasinghe and Williams, 2007) .

The process of biosorption involves physiochemical interactions between metal ions and several anionic ligands like carboxyl, phosphoryl, carbonyl and sulphydryl which are present in the surface of the biomass (Volesky, 1994). The efficiency of the biosorption process depends on the type of metal ion being studied to the type of biosorbent (Tunali et al., 2006). Biosorbent materials, such as the green algae *Spirogyra* species (Gupta and Rastogi, 2008), *Azadirachta indica* bark (King et al., 2008),*Rhizopus oryzae* (Bhainsa and DŠsouza, 2008) and *Bacillus jeotgali* have been reported in biosorption (Green-Ruiz et al., 2008). Dead as well as living microbial cells has been studied for biosorption and the results showed that dead microbial cells is more advantageous in water treatment due to the fact that dead organisms are not affected by toxic wastes, they do not require a continuous supply of nutrients and can be regenerated and reused for many cycles (Aksu, 2005). The maximum adsorption of Pb(II) by *Bacillus cereus* and *Bacillus pumilus* was 28.06 and 22.1 mg/g respectively (Çolak et al., 2011), whereas for *Bacillus licheniformis*, removal of Cr (VI) was tested, maximum biosorption capacity was obtained to be 60.5 mg/g (Zhou et al., 2007). In case of the *Citrobacter* strain MCM B-181 a maximum of 70.8 mg/g of uptake was observed, also a decrease in 13- 68% of Pb(II) uptake was noticed when the biomass was treated with heat and autoclaving (Puranik and Paknikar, 1999). In every study not more than 90% of metal removal was noticed by the bacterial species. Additionally, study of metal removal by a bacterial strain originating from water hyacinth compost has not yet been performed.

Therefore, for the current study, the bacterial species was previously isolated from the water hyacinth compost, which is loaded with huge metal content. The *Bacillus badius* AK strain was most robust and vigorous, surviving in the unstable conditions of composting. However, investigations on the kinetic, isothermal study, desorption and reusability of the biomass is limited. In current study process parameters influencing the biosorption were

standardized in anticipation of utilization of biomass in large scale metal recovery system in future. The aim of this study is to evaluate the metallic sorption potential of bacterial strain *Bacillus badius* AK.

5.2.1 Initial Characterization of the Pretreated (dried) Biomass

• CHNS analysis

Results of C, H, N, S analysis is given in Table 5.3. Biomass has maximum carbon and small amount of hydrogen and sulfur. Carbon is content more than 39%, ash content is 12% and moisture content is 3.14%, this signifies that the exhausted biomass can be used for incineration purpose as shown in Table 5.3.

Table 5.3: CHNS Analysis of *Bacillus badius* AK

Sample	C	H	N	S	Ash	Moisture Content
Bacillus badius AK	39.537	5.898	10.825	0.48	12	3.14

• Brunauer Emmett-Teller (BET) analysis

BET surface area of the adsorbent was carried out and results are given in Table 5.4. The Brunauer-Emmett-Teller (BET) surface area was found 1.060 m^2/g. The Barret-Joyner-Halenda (BJH) surface area was found 1.430 m^2/g in adsorption and 2.126 m^2/g in desorption. The pore diameter were ranging from 0.6182 to 615.5474 nm in adsorption and in desorption it was 1.3435 to 670.8975 nm. The average pore diameter was 2.533 nm. The pore volume was observed as 0.004 cc/g in adsorption. From BET analysis, it was observed that biomass has predominate mesopores and micropores in adsorption, where in desorption micropores are predominant.

Table 5.4: The results of Brunauer Emmett-Teller (BET) measurements

Parameter	Values
Surface area (m^2/g)	1.06
Pore volume (cm^3/g)	0.004
Pore size (nm)	2.533

5.2.2 Batch Biosorption Studies

• Effect of pH

The pH is an important parameter which influences the metal microbe interaction. In the present study the pH was varied as 3, 4, 5 and 6. The change in initial concentration of Pb(II) at different pH values was observed. Many authors have reported that the surrounding pH value is one of the most important factors in biosorption efficiency by different microorganisms (Al-Asheh and Duvnjak, 1995). The lower metal uptake at lower pH may be attributed to the higher concentration of H^+ ions competing for metal binding sites on the biomass. However at higher pH, solubility of Pb(II) was lowered. The increase in the adsorption at higher pH may be due the decreased ionic competition and also the decreased solubility of the metals at higher pH, which may induce increased adsorption onto the biomass up to a particular pH beyond which the precipitation initiates (Puranik and Paknikar, 1999). With the increase in pH there is an increase in ligands with negative charges which results in increased binding of the cations (Gong et al., 2005). The results from the present work are in accordance with the past studies. The efficiency of adsorption by *Bacillus badius* AK increased from pH 4-6 (Figure 5.10). Other bacteria of the same genus *Bacillus* have also shown higher removal within this pH range of 4-6 (Singh et al., 2012; Çolak et al., 2011; Oves et al., 2013). The optimum pH for Pb removal was found to be 5. It was observed that precipitation of lead started at pH > 5.65 thereby it can be concluded that pH 5.65 is the critical value for precipitation of lead, beyond which initial metal concentration started decreasing. Significant differences were observed between all the triplicates ($p < 0.05$). Similar observation was also made by other authors where there was decrease in metal adsorption beyond pH 6. This fact is also supported by distribution of lead species at different pH (Singh et al., 2012).

• Effect of contact time

The contact time also plays a vital role in the adsorption process. The increase in the contact time had a significant effect on the biosorption of metal ions. Since a large no. of vacant sites for the biosorption are available initially, the biosorption in the initial stages are rapid and increases up to 30 min and then increases slowly, becoming constant thereafter (Çolak et al., 2011). The equilibrium time for biosorption by *Bacillus subtilis* was found to be 100 min (Singh et al., 2012). The optimum biosorption capacity of *Citroabacter freundii* at pH 4 with initial lead concentration of 481.21 mg/L and contact time with biomass dosage of 2 g/L was observed to be 100 min (Al-Garni, 2005). The equilibrium time for

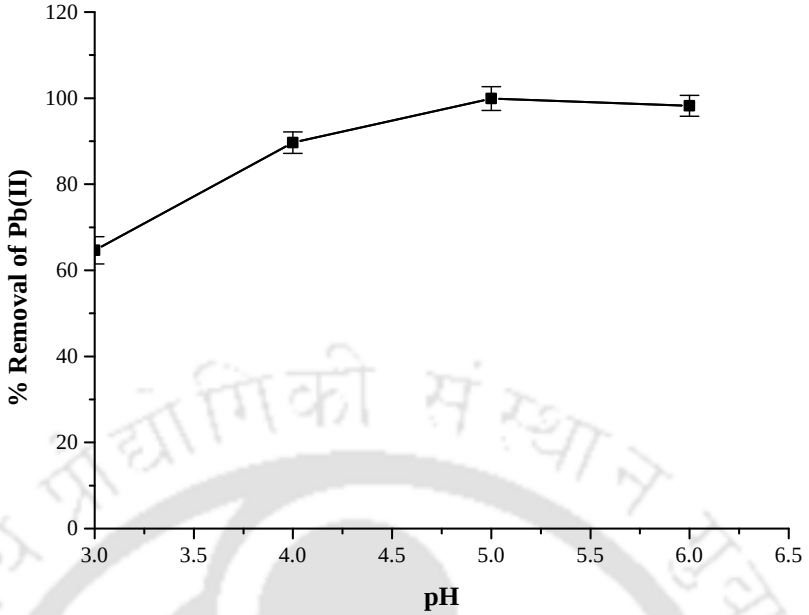


Figure 5.10: Effect of pH on percentage removal of Pb(II) by *Bacillus badius* AK at constant agitation of 150 rpm , initial biomass dosage of 2 g/L and initial metal concentration of 100 mg/L

lead biosorption (concentration 75 mg/L) by *Bacillus cereus* and *Bacillus pumilis* was 80 min (Çolak et al., 2011). In the present study, the change in metal removal efficiency was

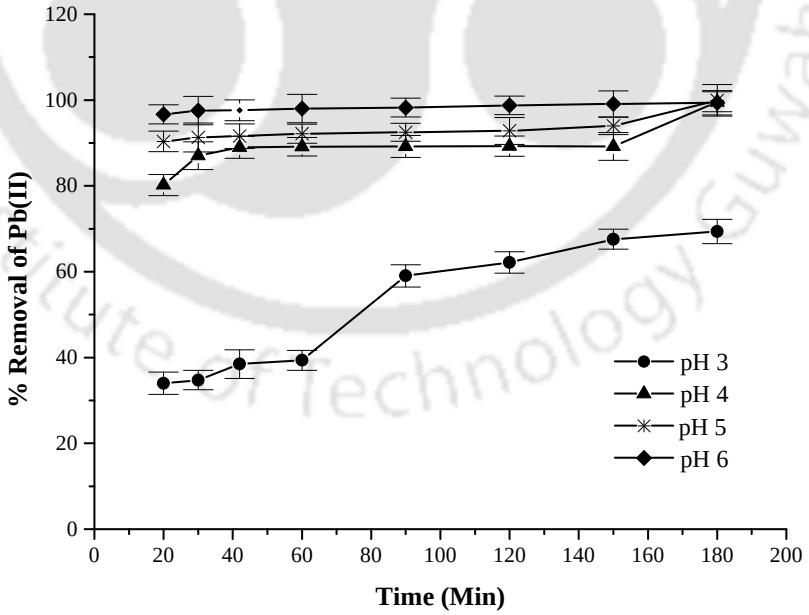


Figure 5.11: Effect of contact time on percentage removal of Pb(II) by *Bacillus badius* AK at constant agitation of 150 rpm , initial biomass dosage of 2 g/L and initial metal concentration of 100 mg/L

observed for a time period of 3 h. Observations were made after every 15 min for the first 1 h and in the interval of 30 min thereafter. Almost 90% of Pb(II) removal was achieved in the first 30 min within pH range of 4-5 (Figure 5.11). On analyzing the results by ANOVA, a significant variance was observed among all the trials ($p < 0.05$). Similarly, it was reported that the biosorption of lead by the fungus *Phanerochaete chrysosporium* was rapid in the first 15 min and equilibrium was attained after 3 h (Çeribasi and Yetis, 2001). The rate of metal uptake is usually influenced by the factors affecting mass transfer from bulk solution to the binding sites there are three major steps. First is the bulk transport of the metal ions in the solution phase which is rapid due to the advection flow. Second film transport which involves diffusion of metal through a hydrodynamic boundary layer around the biosorbent surface and third the actual adsorption of the metal ions by active sites of the biomass is considered to be rapid and equivalent to an equilibrium reaction.

- **Effect of temperature and thermodynamics study**

The temperature was varied from 20 to 50°C by keeping samples in the temperature controlled shaking incubator. The pH was adjusted at 5 with biomass dosage of 2 g/L at 100 mg/L of Pb(II), rotational speed of 150 rpm for 180 min. The percentage removal was found to increase both in case of 30°C and 40°C, a maximum of 98.8% removal was achieved at 40°C, whereas 97.8% of maximum removal was observed at 30°C. Thus, 40°C was concluded as the optimum temperature for Pb(II) removal. At 20°C, the maximum removal was 87% up to 1.5 h and then decreased thereafter. Much less removal was observed at 50°C where maximum removal was 52% within 1 h and then started decreasing (Figure 5.12a). The Pb(II) metal uptake by *Bacillus badius* AK was found to increase from 20-40°C, with the maximum removal at 40°C and then was found to decrease (Figure 5.12b). The increase in metal uptake at higher temperature is due to higher affinity of sites for metal or an increase in binding sites on the biomass (Marques et al., 1991). An increase in metal uptake was observed when the temperature was increased from 4-25°C, but a decrease in metal uptake was observed when the temperature was further increased up to 50°C, while studying Cu^{2+} biosorption on *Aspergillus carbonarius*, they attributed the increase in metal adsorption to the increase in energy of the system that facilitated attachment to the cell surface and the decrease in metal sorption due to the distortion of adsorption sites on the cell surface (Al-Asheh and Duvnjak, 1995). The observed trend with increasing temperature suggests that the biosorption of Pb(II) with the biosorbent is kinetically controlled by an endothermic process (Zhang et al., 2013). ANOVA analysis with ($p < 0.05$) depicted significant differences among all the triplicates. From this study the optimum temperature was found to be 40°C and contact time of 2.5 h.

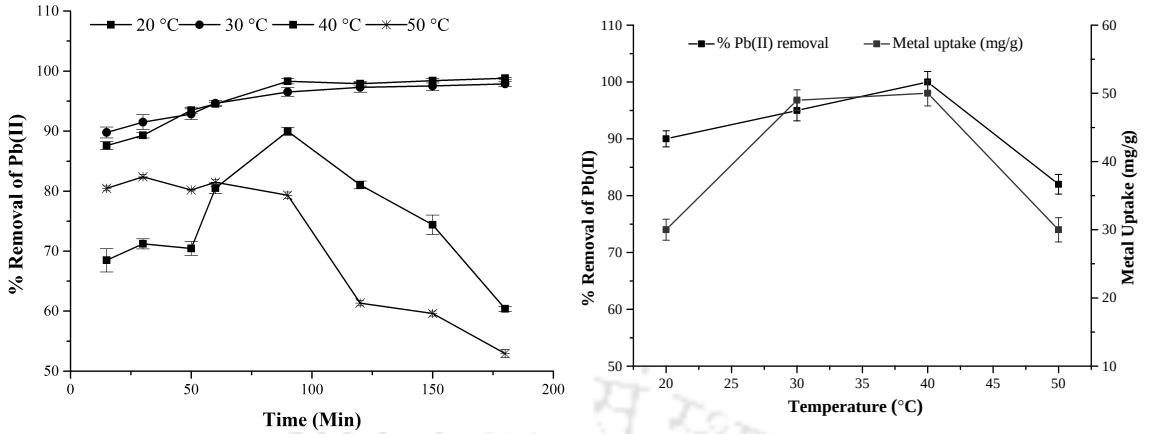


Figure 5.12: ((a) Change in removal percentage of Pb(II) with time at different temperatures (b) Effect of temperature on removal and uptake of Pb(II) at optimum conditions of pH 5, temperature 40°C, biomass dosage 2g/L, rotational speed of 150 rpm.

The Thermodynamic parameters such as the free energy change, enthalpy change and entropy change were calculated (Eq. 3.14 and Eq. 3.15). Table 5.5 summarizes the thermodynamic parameter of Pb(II) solution with initial concentration of 100 mg/L and biomass dosage of 2 g/L. Positive value of ΔH° concluded the endothermic behaviour of adsorption and this was supported by the increase in the uptake with increase in temperature, negative ΔG° value indicated the process is spontaneous and feasible.

Table 5.5: Thermodynamics parameters for biosorption of Pb(II) (100 mg/L)

Biomass dosage (g/L)	**Temperature (°C)**	ΔH° **(J/mol)**	ΔS° **(J/mol.K)**	**-**ΔG° **(J/mol)**
	20			106023.76
2	30	102.6	362.21	109645.82
	40			113267.88
	50			117017.20

The low value of ΔS° implied that no remarkable change in entropy occurred during the adsorption process but there is randomness at solid-solution interface. The biosorption heat for Pb(II) was found to be 0.102 KJ/mol. The magnitude of the heat, ΔH° value gives an indication on the type of adsorption, which can either be physical or chemical. If the heat of adsorption is less than 20.9 KJ/mol, then it is physical adsorption and the heat of chemisorption falls in the range of 20-200 KJ/mol which is similar to the heat of chemical reactions(Goswami and Purkait, 2012), suggesting that physical adsorption was dominant onto *Bacillus badius* AK at a dose of 2 g/L. Similar results were found when *Bacillus gibsonii* S-2 waste biomass was used for removal of Pb(II) from aqueous solution, the ΔH° and ΔS° were 23.1 KJ/mol and 87.46 J/mol/K and ΔG° being -2.57 KJ/mol (Zhang et al., 2013).

• **Effect of initial biomass dosage**

The initial biomass dosage was varied as 0.5, 1, 2, 3 and 4 g/L with an initial metal concentration of 100 mg/L. The test was conducted at an optimum temperature of 30°C, pH 5 and rotational speed of 150 rpm for 3 h. The optimum dosage was found to be 2 g/L (Figure 5.13). Significant differences were observed between all the triplicates ($p < 0.05$). The removal efficiency increased up to 2 g/L and then started decreasing. Similar observation was made when *Bacillus cereus* and *Bacillus pumilis* bacteria were used as a biosorbent and Pb(II) metal uptake increased upto 1g/L, then started decreasing (Çolak et al., 2011). The ratio of the metal concentration to the amount of biomass concentration was found to be an important factor in the biosorption of the metals (Deng et al., 2007). A reduction in zinc uptake by *Rhizopus arrzihus* was reported with increasing biomass concentration and is attributed to an insufficiency of metal ions in the solution with respect to the binding sites (Fourest and Roux, 1992).

A similar observation was made in study of metal sorption by *Phormidium laminosum* biomass (Sampedro et al., 1995). Higher uptake at lower biomass concentrations can be due to an increased metal-to-biosorbent ratio, which decreases upon increase in biomass (Puranik and Paknikar, 1999).

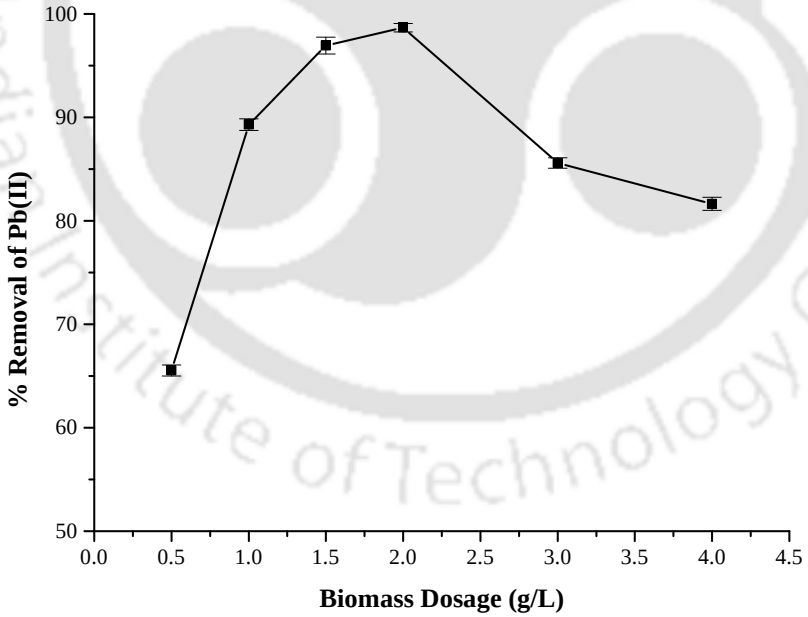
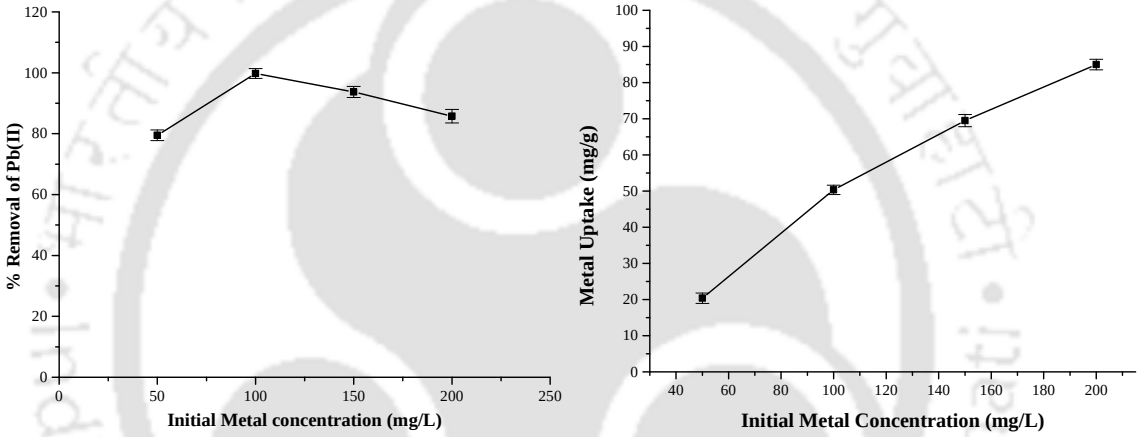


Figure 5.13: Change in removal percentage of Pb(II) at varying initial biomass dosage

• **Effect of initial metal concentration**

The initial metal concentration was varied as 50, 100, 150 and 200 mg/L with initial biomass dosage of 2 g/L, agitation speed of 150 rpm, pH 5 and temperature 40°C for 2.5 h. The percentage removal was less at 50 mg/L, reached maximum at 100 mg/L and then decreased thereafter (Figure 5.14a). The specific metal uptake was observed to be increasing with the increase in initial metal concentration (Figure 5.14b). Similar enhancement in metal uptake was observed during the biosorption of Pb(II), Cd(II) and Zn (II) by *Citrobacter* strain MCMB-181, the observed enhancement in the metal sorption could be due to the increase in the electrostatic interaction, involving sites of lower affinity for metal ions (Puranik and Paknikar, 1999).



(a) (b)

Figure 5.14: (a) Change in Pb(II)% removal with initial metal conc. (b) Change of metal uptake with initial metal conc. (Initial biomass dosage of 2 g/L, pH 5, temp. 40°C and 2.5 h of contact time with an agitation speed of 150 rpm.

• **Kinetic study**

The adsorption kinetics is the rate of adsorption of adsorbate towards adsorbent. The adsorption kinetics can be well treated and explained by LagergrenâĂŹs pseudo first and second order kinetic models. The adsorption kinetics onto *Bacillus badius* AK was analysed using Lagrange's pseudo first order and second order kinetics. The experimental data obtained were fitted into pseudo-first-order kinetic model using method of least squares and the obtained parameters are listed in Table 5.6.

Similarly, the obtained experimental data were fitted with of pseudo second and first order model as shown in Figure 5.15. The initial metal concentration was kept at 100 mg/L, biomass dosage 2 g/L, temperature 40°C and an agitation of 150 rpm. From kinetics

Table 5.6: Comparison of pseudo first order and second order kinetics model

Initial conc. of Pb(II) (mg/L)	q_e,**exp (mg/g)**	**Pseudo first order kinetics**		**Pseudo second order kinetics**		
		k_1 (/min)	q_e,cal (mg/g)	R^2	k_2 (g/mg.min)	q_e,cal (mg/g)
100	48.94	0.00384	32.6	0.999	0.0074	50.51

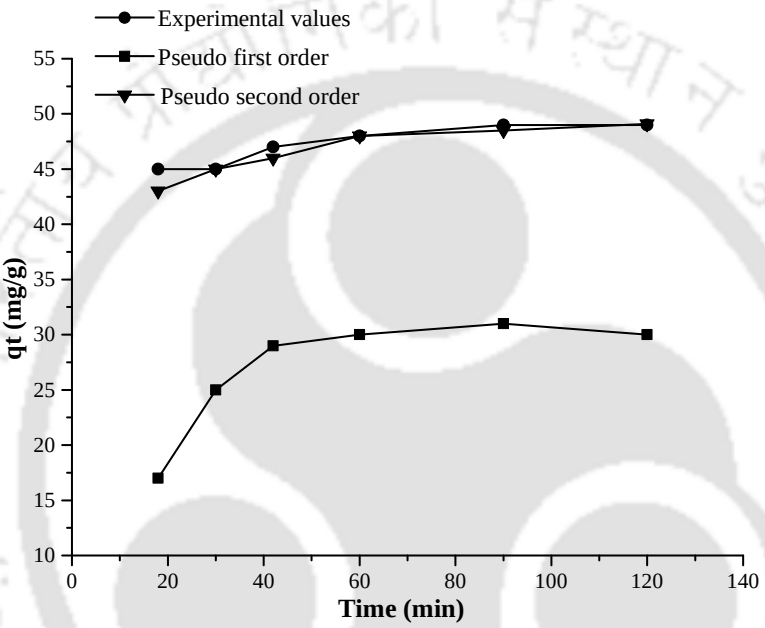


Figure 5.15: Plots of pseudo first order, second order and experimental data for Pb(II) biosorption

study it was observed that equilibrium was reached after 2.5 h. The values of rate constant, capacities, and correlation coefficient from pseudo first and second order are summarized in Table 5.6. The R^2 value was 0.99 in pseudo second order and moreover the equilibrium adsorption capacity calculated using pseudo second order model was in close agreement with the experimentally obtained values. These results suggest that pseudo second order kinetic model is better than the pseudo first order kinetic model in representation of the kinetics of the biosorption system. The pseudo second fitted better for the biosorption system when *Bacillus gibsonii* S-2 waste biomass was used for removal of Pb(II) from the aqueous solution (Zhang et al., 2013).

• **Isotherm study**

The Langmuir and freundlich isotherm models were adopted to predict the behaviour of metal uptake by dried *Bacillus badius* AK. The results are shown in Table 5.7. The initial metal concentration was 100 mg/L. From the results it can be seen that Langmuir model fitted better than Freundlich model (Figure 5.16a, 5.16b, and 5.16c), as the R^2 value is higher for Langmuir model. Conformity of the data to the Langmuir isotherm indicated that biosorption of metal ions could be characterized as a monolayer single type phenomenon with no interaction between the metal ions (Figure 5.16b). The maximum adsorption capacity q_{max} was 138.88 mg/g and the constant K_L (which is the ratio of adsorption rate constant to the desorption rate constant) showed a high value, thereby indicating high affinity of the metal ions for the binding sites on the adsorbent (Tunali et al., 2006).

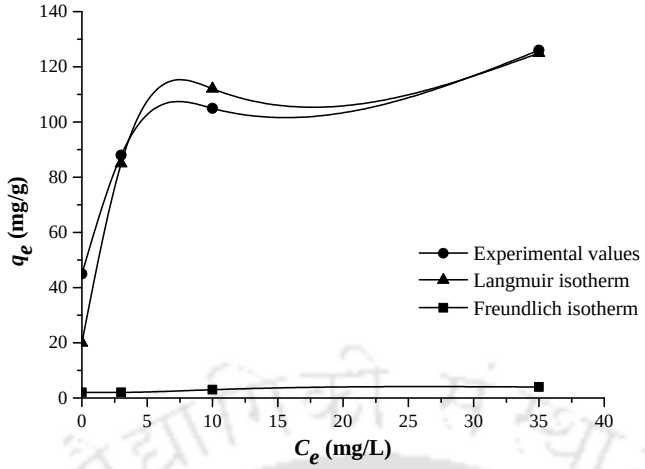
The equilibrium parameter R_L which is defined as the R_L=1/(1+b.C_o) in the range of 0< R_L<1 reflects a favourable adsorption process where b (L/mg) is the Langmuir's constant and C_o (mg/L) is the initial adsorbate concentration. The R_L value was found to be as 0.02 therefore indicating that adsorption process was favourable and Langmuir's isotherm was applicable. A comparative study of the isotherms on the existing literature of microbial biomass is listed in Table 5.8.

Table 5.7: Comparison of Langmuir and Freundlich isotherms

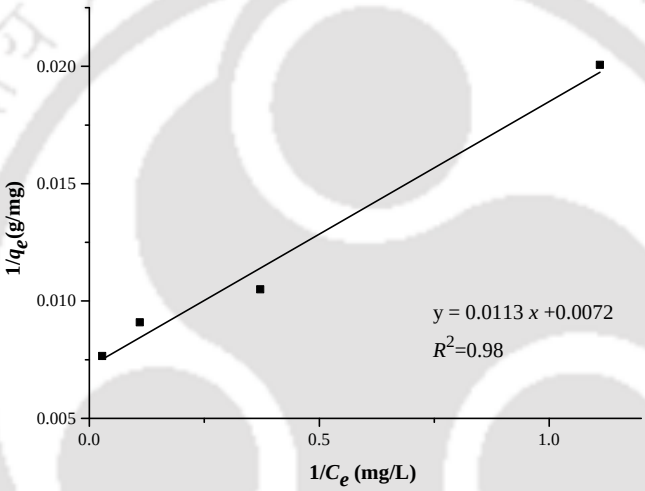
	Langmuir isotherm			**Freundlich isotherm**		
Initial concentration of Pb (mg/L)	R^2	q_m (mg/g)	K_L (L/mg)	R^2	n	K_F (mg/g)
100	0.985	138.88	0.63	0.947	4.94	6.27

Table 5.8: Comparison of Isotherms obtained from different Biomass

Type of Dry Biomass	**Suitability of Isotherms**	**Authors**
Streptoverticillium cinnamoneum	Langmuir & Freundlich	(Puranik and Paknikar, 1997)
Citrobacter sp.	Langmuir	(Puranik and Paknikar, 1999)
Bacillus Strain from soil	Langmuir	(Tunali et al., 2006)
Bacillus cereus and *Bacillus pumilus*	Langmuir	(Çolak et al., 2011)



(a)



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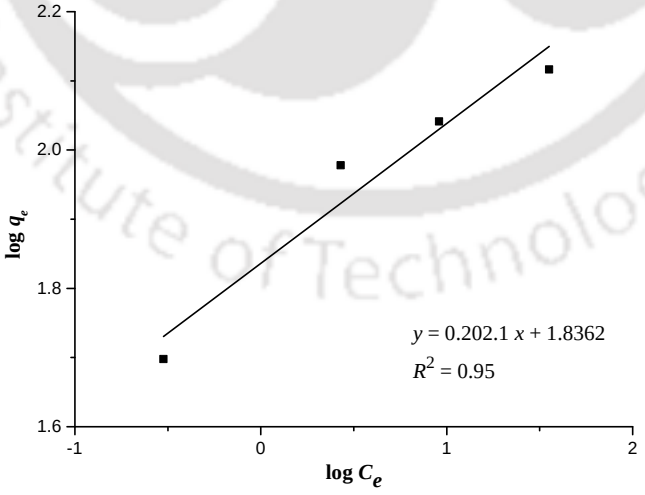


(c)

Figure 5.16: (a) Plot of q_e (mg/g) against the final concentration C_e (mg/L) using experimental, langmuir and Freundlich iostherms for biosorption of Pb(II) (b) Langmuir isotherm plot (c) Freundlich isotherm plot

• **XRD analysis**

The XRD pattern of *Bacillus badius* AK before and after the biosorption of Pb(II) is shown in Figure 5.17. Sharp intensity XRD peaks have been observed at typical scanning angles of $2\theta = 20°$ to $60°$ for dried bacterial biomass before and after the Pb(II) adsorption.

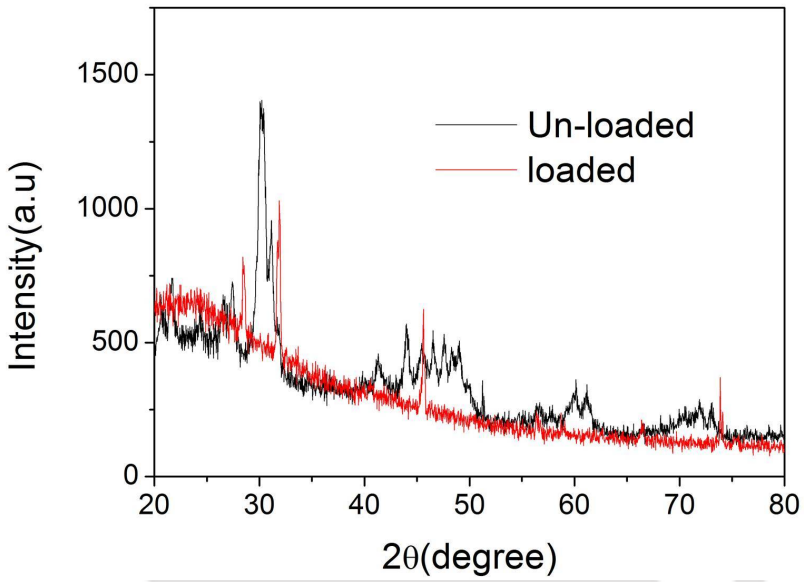


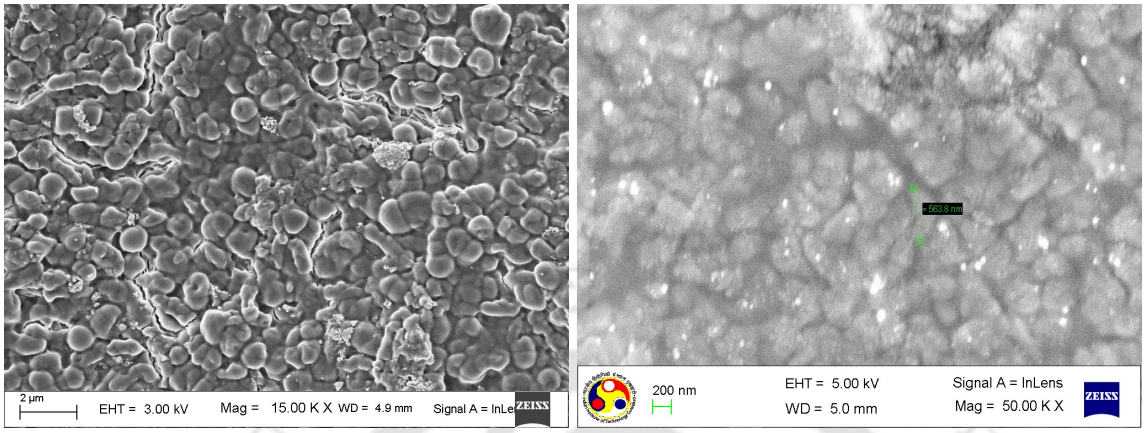
Figure 5.17: XRD analysis of the *Bacillus badius* AK

The sharp peaks depicted the morphology of dried biomass, while the low intensity peaks within that range showed the change in topology of bacteria after the Pb adsorption. The XRD profile of Pb(II) free *Bacillus badius* AK demonstrated a humped peak between 25° and 34° and other distinct peaks were observed between 40° to 50°, 59° to 61°. The sharp peaks are attributed to the native crystalline structure of dried biomass but Pb(II) loaded cells showed low intensity peak indicating amorphous structure of biomass due to Pb(II) stress.

• **SEM and EDX analysis**

SEM micrographs indicated the topology of *Bacillus badius* AK, as rod and regular in shape before after Pb(II) biosorption, its surface became rough and porous. (Figure 5.18a and 5.18b). The observation from SEM was confirmed by EDX spectra which revealed the presence of Pb(II) after metal loading. This observation was confirmed by the EDX analysis, the peaks between 2 and 3 KeV was corresponding to Cl^- (Figure 5.19), it disappeared after Pb(II) biosorption, significant appearance of peaks for Pb(II) were observed within 2 to 3 KeV range (Figure 5.20). The EDX analysis in the biomass loaded with Pb(II) shows a decrease in intensity of Cl^- bands and no significant decrease in intensity was observed

for Mg^{2+} and K^{+}, these findings indicate that the biosorption process might include ion exchange.



(a) (b)

Figure 5.18: (a) & (b) Typical SEM micrographs of *Bacillus badius* AK before and after biosorption.

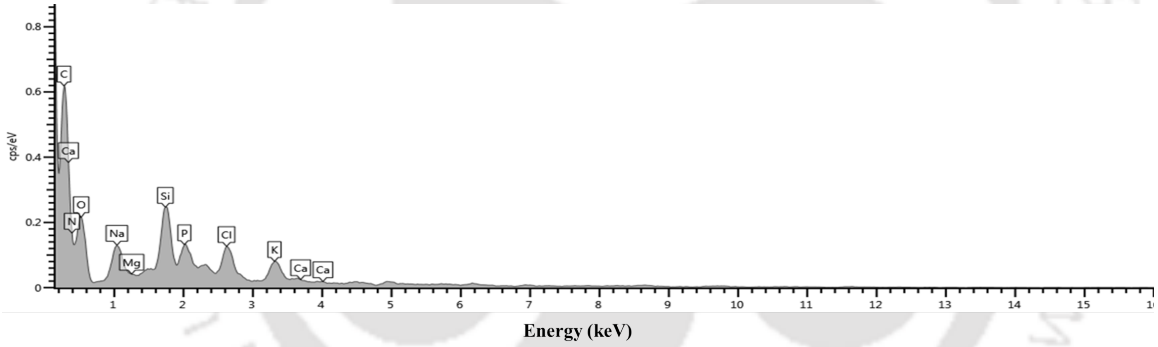


Figure 5.19: Typical EDX spectra of *Bacillus badius* AK before Pb(II) biosorption

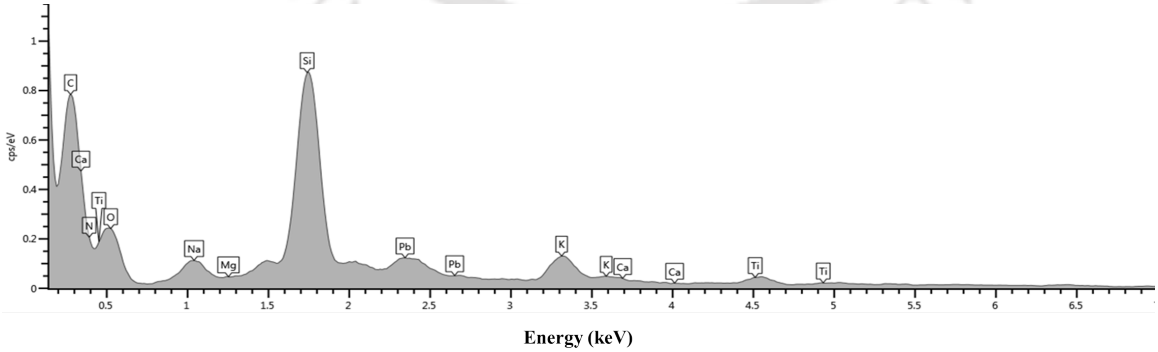


Figure 5.20: Typical EDX spectra of *Bacillus badius* AK after Pb(II) biosorption

• **FTIR spectral analysis**

The Fourier transform infrared spectroscopy (FTIR) spectra of loaded and metal loaded *Bacillus badius* AK biomass in the range of 400-4000 cm^{-1} were taken to find out which functional groups are responsible for the biosorption process and presented in Figure 5.21. As seen from the figure, unloaded biomass displays a number of absorption peaks, reflecting the complex nature of the biomass and the presence of functional groups responsible for biosorption of Pb(II).

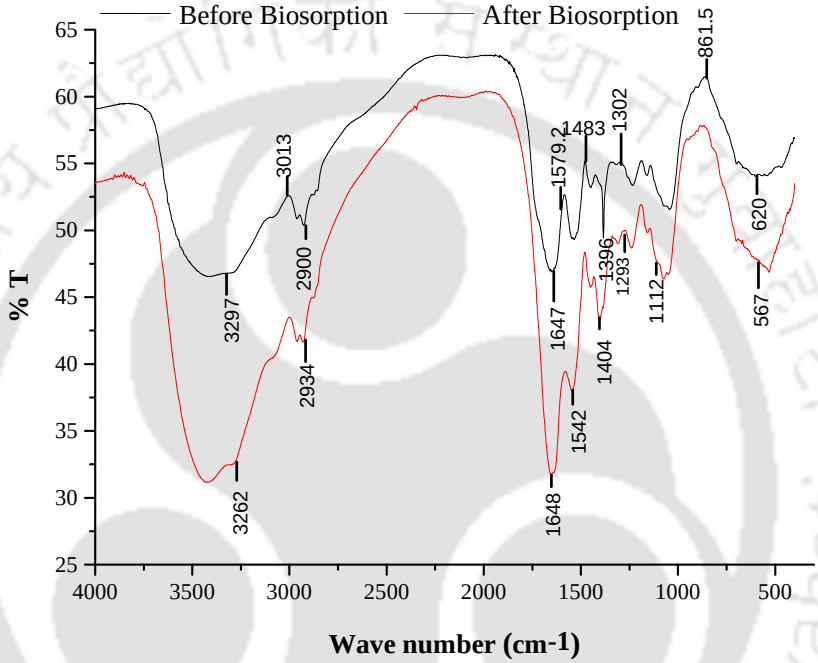


Figure 5.21: FTIR spectra of *Bacillus badius* AK before and after biosorption

Table 5.9: FTIR Characteristics of dried *Bacillus badius* AK biomass

Wave number	**Biomass before biosorption**	**Biomass after biosorption**	**Difference**	**Assignment**
3700-3600	3297.45	3262.4	-35.05	Surface hydroxyl group (OH)
2990-2850	2934.65	2900.6	-34.05	CH_3 and CH_2 in aliphatic compounds
1650-1620	1647.5	1648.8	1.3	C=O groups
1590-1545	1579.24	1542.56	-36.68	NH_2 primary alkyl amide
1420-1370	1396.2	1404.34	8.13	C–N in primary amide
1335-1295	1302.1	1293.11	-5.29	$-SO_2$ stretching
630-565	620.1	567.4	-52.7	C–CO–C bend

It indicated the probable involvement of the functional group in the adsorption process. The FTIR spectra on the unloaded biomass and loaded biomass, respectively, indicating

bounded hydroxyl (-OH) or amine (-NH) groups. Peak at 2929 cm^{-1} is referred to the (CH) of cellulose and hemicelluloses (Weng and Wu, 2011). The peak appeared with a wave number of 1648 cm^{-1} was carboxylic groups (Mohanty et al., 2000). The peaks at 2990 cm^{-1} and 2850 cm^{-1} are CH_3, symmetric bending. Peak at the 1396 cm^{-1} and 1334 cm^{-1} are referred to the SO_3 stretching. The peaks at 1074 $^{-1}$ and 1044 cm^{-1} may be explained as due to O-H alcohols. Transmittance of peaks in loaded biomass was substantially lower than the unloaded biomass. These changes suggest that bond stretching occurs due to presence of metals and therefore, peak transmittance is reduced. Consequently, the formation of varying spectra followed by the adsorption of Pb(II) on bacterial biomass validated the contribution of functional groups in Pb(II) binding. In agreement to present work other researchers have reported similar observation (Dekhil et al., 2011; Oves et al., 2013). These changes observed in the spectrum indicated the possible involvement of functional groups in the biosorption process on the surface of the biomass.

• **Desorption, recovery and reuse**

Lead was desorbed by treatment with different desorbents. The initial metal concentration was measured after biosorption, the biosorbent was dried and contacted with 0.1M EDTA, HCl, HNO_3, CH_3COOH and H_2SO_4 and Distilled water (Deng et al., 2007). The percentage of Pb(II) released is shown in Figure 5.22. The initial pH of each desorbent was noted and a total desorption time of 3.5 h was provided initially. It was found 0.1M HCl, H_2SO_4 and HNO_3 gave more 98% recovery of Pb(II). But desorption with HCl was achieved within 2.5 h as compared to 3.5 h, each for H_2SO_4 and HNO_3, again the recovery with HNO_3 nitrify the biosorbent. The recovery with 0.1M CH_3COOH and EDTA was 96 and 88% respectively, whereas negligible recovery was observed with distilled water. The recovery rate of Pb(II) by HCl makes the biosorbent suitable for reuse.

The recovery of biosorbent after biosorption was 42% of the initial value and the recovery after desorption was 71.4% (all these values are reported after considering the losses during recovery).

The biosorbent obtained after desorption was reused for studying the biosorption of *Bacillus badius* AK with initial metal concentration of 100 mg/L of Pb(II), temperature 40°C, biomass dosage of 2 g/L, pH 5 and agitation speed of 150 rpm. ANOVA analysis with ($p < 0.05$) depicted significant differences among all the triplicates. Results indicated that percentage removal of Pb(II) was 60%, this might be due the destruction of sites at the surface of the biosorbent during desorption. Similar decrease in biosorption after desorption with acid (0.1M HCl) was observed during biosorption of Pb(II), Cd(II) and Zn (II) by *Citrobacter* strain MCMB-181, they attributed the decrease to the deleterious effect of acid on the

Table 5.10: Comparison of Pb(II) uptake by different dried biomass

Type of Biomass	pH	Contact time (min)	Lead uptake (mg/g)	Author
Streptoverticillium cinnamoneum	3.5-4.5	75	57.7	(Puranik and Paknikar, 1997)
Bacillus cereus and *Bacillus pumilus*	6	80	22.1, 28.06	(Çolak et al., 2011)
Citrobacter sp.	4.5	80	70.8	(Puranik and Paknikar, 1999)
Delftia tsuruhatensis	6	20	22.38	(Bautista-Hernández et al., 2012)
Citrobacter freundii	4	100	58.5	Al-Garni (2005)
Rhizopus arrhizus	05	-	85.6	(Fourest and Roux, 1992)
Bacillus badius AK	5	150	138.88	Present study

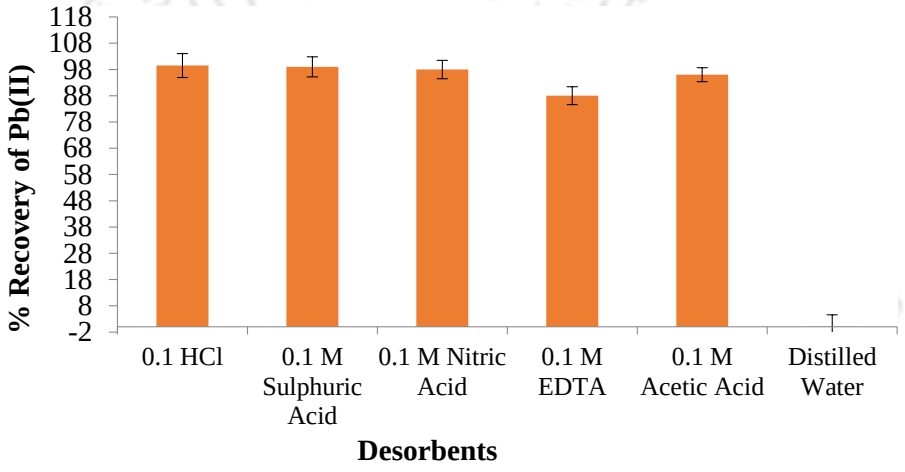


Figure 5.22: Percentage recovery of Pb(II) with different desorbents

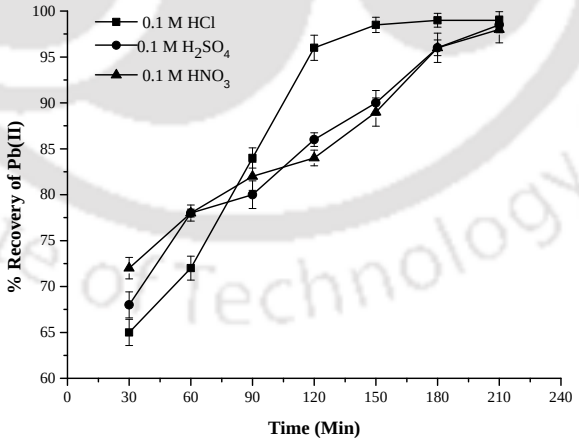


Figure 5.23: Percentage recovery of Pb(II) with time using different desorbents

biomass (Puranik and Paknikar, 1999). A comparison of maximum uptake of heavy metals by the microbial biomasses in existing literature and *Bacillus badius* AK has been presented in the Table 5.10.

• **Intraparticle diffusion model**

To determine the adsorption process mechanism of Pb(II) by *Bacillus badius* AK, intraparticle diffusion model proposed by Mark and Webber (1963) was used in terms of graphical relationship between the amount of Pb(II) adsorbed (q_t) and and square root of time ($t^{0.5}$) (Eq. 3.17). Figure 5.24, 5.25, 5.26 and 5.27 show the plots of intraparticle diffusion models at different initial concentrations of Pb(II). In Figure 5.25 at optimum initial Pb(II) concentration of 100 mg/L, biomass dosage of 2 g/L, pH 5, agitation speed of 150 rpm and contact time of 2.5 h it can be observed that the the plot has initial curved portion followed by a linear portion which suggests surface adsorption and intraparticle diffusion both were responsible for adsorption of Pb(II), where the initial curved portion of the graph is due to boundary layer effect and the linear portion is due to intraparticle diffusion and the effect of boundary layer diffusion is more than that of intraparticle diffusion where k_d was 0.9 mg/g/min$^{0.5}$.

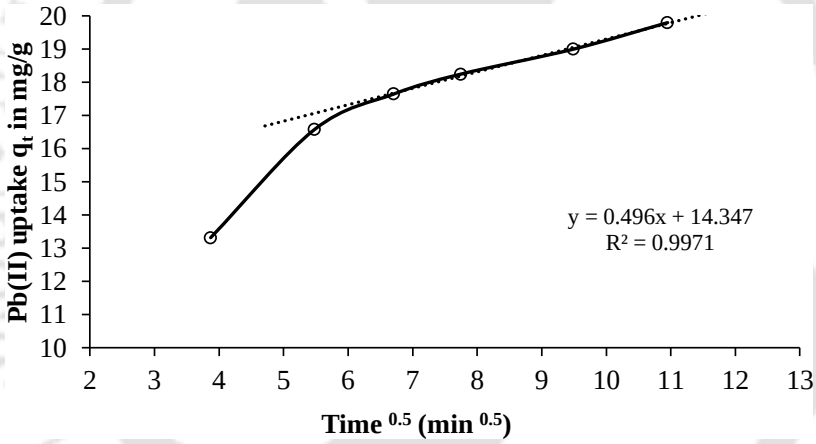


Figure 5.24: Variation of q_t with time 0.5 at 50 mg/L of Pb(II)

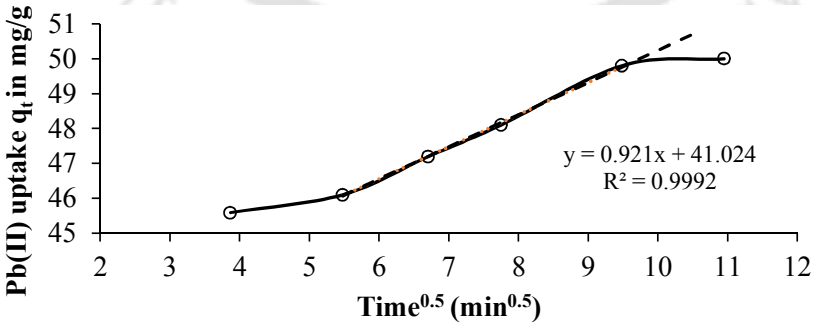


Figure 5.25: Variation of q_t with time 0.5 at 100 mg/L of Pb(II)

Figure 5.24 suggests that the biosorption is mainly due to intraparticle diffusion with k_d was 0.5 mg/g/min$^{0.5}$, for initial concentration of 150 mg/L the plot suggests that sorption

was effected both due to intraparticle diffusion and boundary layer and for 200 mg/L both boundary layer effect was mainly responsible for biosorption where intercept was 77.27 mg/L and k_d 0.8 mg/g/min$^{0.5}$. The slope of the linear portion of the plot has been defined as a rate parameter which characterizes the rate of adsorption in the region where intraparticle diffusion is rate limiting. The intercept value of the plot, on the other hand, signifies the extent of boundary layer effect, i.e. larger the intercept, the greater is the contribution of the surface adsorption in the rate limiting step (Sarkar et al., 2003). The intraparticle diffusion parameter (k_d) was calculated from the slopes and the intercept values are summarized in Table 5.11. The high intercept value suggests that the adsorption process occurred mostly due to surface adsorption process and very little adsorption occurred through intraparticle diffusion.

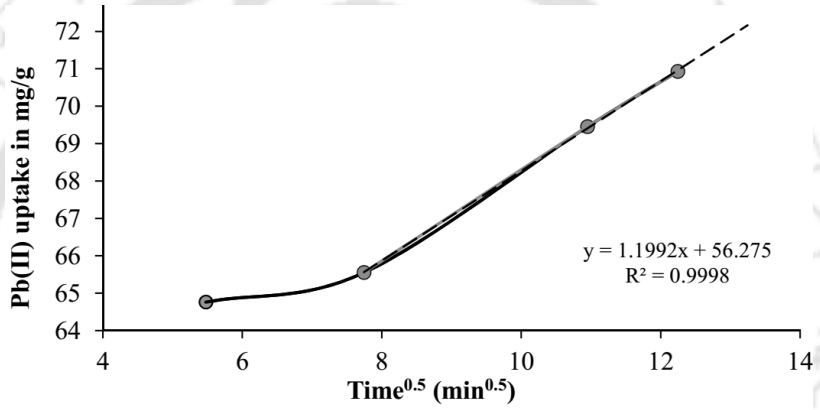


Figure 5.26: Variation of $q_t$with time 0.5 at 150 mg/L of Pb(II)

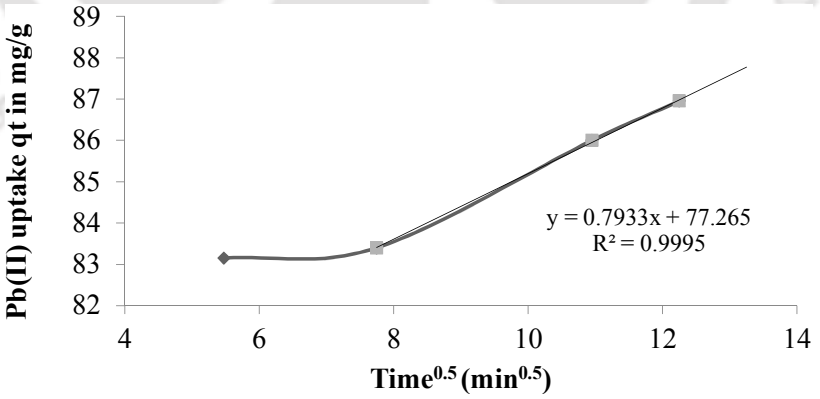


Figure 5.27: Variation of q_t with time 0.5 at 200 mg/L of Pb(II)

Table 5.11: Intraparticle diffusion values and intercepts values for different initial concentrations

Initial concentration of Pb(II) in mg/L	k_d **(mg/g.min$^{0.5}$)**	**C*(mg/L)**
50	0.5	14.4
100	0.9	41.0
150	1.2	56.3
200	0.8	77.3

C* is the intercept of the graph

• **Effect of storage on dried biomass**

The dried biomass of *Bacillus badius* AK was stored under room temperature (25 ± 5°C) and biosorption was carried out at the end of 3, 2 and 1 month at an biomass dosage of 2 g/L, initial metal concentration of 100 mg/L, pH 5, rotational speed of 150 rpm and contact time of 2.5 h at 40°C (Figure 5.28). The data reveals that specific lead uptake was almost similar with very little change at the end of each time period which indicated that the biomass had the same capacity to biosorb Pb(II) ions (Figure 5.29). Similar observations were made when biosorption of Pb(II) was done by gram negative bacteria for a time period of 125 days (Al-Garni, 2005). These findings can be considered advantageous while applying the biosorbent over other conventional methods, as the biosorbent can be safely stored for long periods with very little change in the metal uptake.

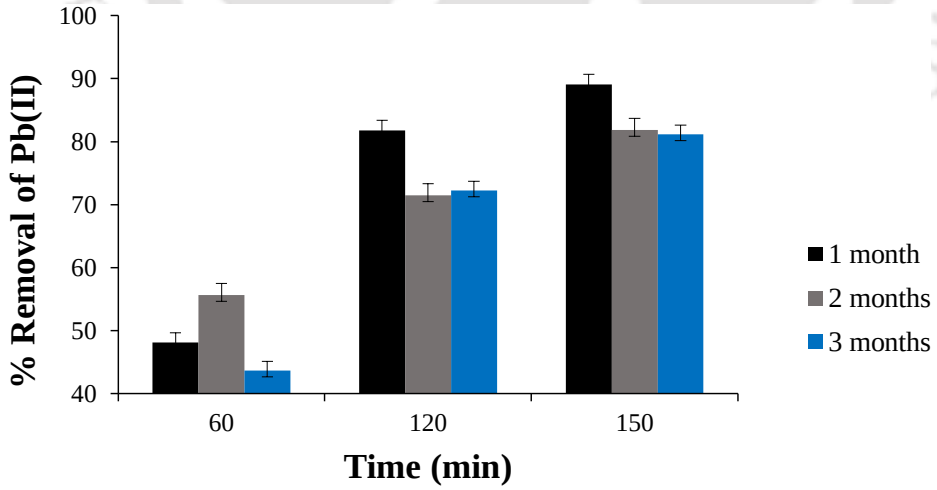


Figure 5.28: Change in removal percentage with storage (at initial metal conc. 100 mg/L, temperature 40°C, initial biomass 0.1 g/L, pH 5, time 150 min, 150 rpm)

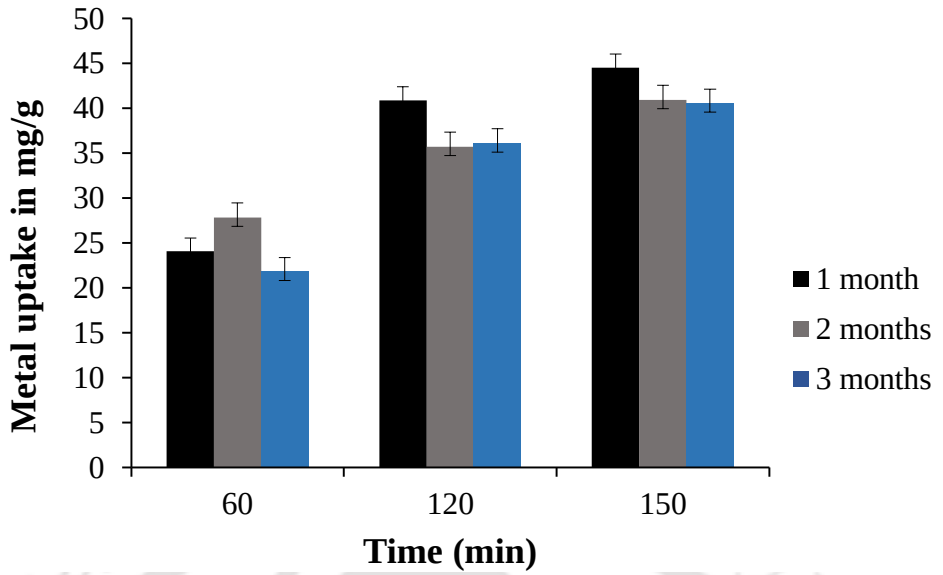


Figure 5.29: Change in uptake of Pb(II) with storage (with initial metal conc. 100 mg/L, temperature 40°C, initial biomass 0.1 g/L, pH 5, time 150 min at 150 rpm)

• **Effect of other anions on the removal efficiency of Pb(II)**

The anions of 0.1, 1 and 10 mM concentration of nitrate, phosphate, chloride and sulphate were added to synthetic solutions of lead having initial concentration of 100 mg/L. The final concentrations were observed after carrying out the biosorption with 2 g/L of biomass dosage, pH 5, 2.5 h of contact time, agitation speed of 150 rpm and temperature 40°C. The percentage removal and metal uptake with respect to different anion concentration are shown in Figure 5.30 and Figure 5.31 respectively. The results indicate that the metal biosorption by *Bacillus badius* AK remained almost unchanged at 0.1 mM concentration for SO_4^{2-}, PO_4^{3-} and Cl^- , the percentage removal was 100% and metal uptake at 50 mg/g except in case of NO_3^- the removal of lead was 95% and uptake 47.5 mg/g. For SO_4^{2-}, PO_4^{3-} and Cl^- at 1 mM and 10 mM the percentage removal and uptake were almost similar except for NO_3^- the removal rate decreased continuously from 85% at 1mM to 51.54% at 10 mM and correspondingly the uptake decreased to 24.23 mg/g at 10 mM from 42.5 mg/g at 1 mM.

The results showed that the biomass can be used successfully even at very concentration of Phosphate (950 mg/L), Nitrate (62 mg/L), Sulphate (960 mg/L) and Chloride (350 mg/L) which are generally the major industrial pollutants. Tobin (1987) described three possible ligands, viz. (i) decrease in metal sorption due to the formation of complexes that are non-adsorbing or weakly adsorbing, (ii) ligands that may interact with adsorbent so as to enhance or decrease the metal uptake potential and (iii) formation of complexes that are more strongly adsorbing than free metal, resulting in enhanced metal uptake capacity.

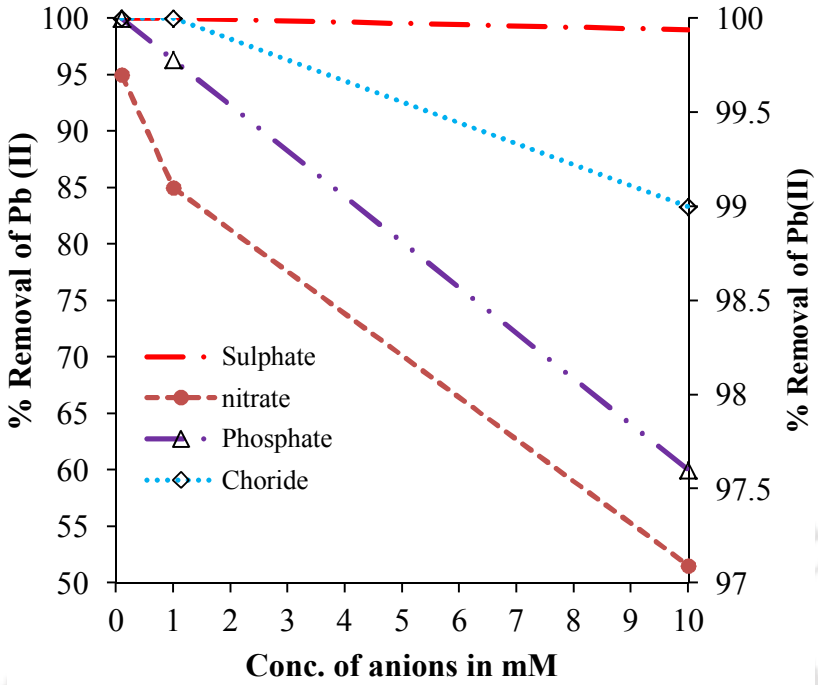


Figure 5.30: Change in removal percentage of Pb(II) at different anion conc. (Biomass dosage of 2 g/L, initial metal conc. Of 100 mg/L, pH 5, temp. 40°C, 150 rpm and contact time of 2.5 h)

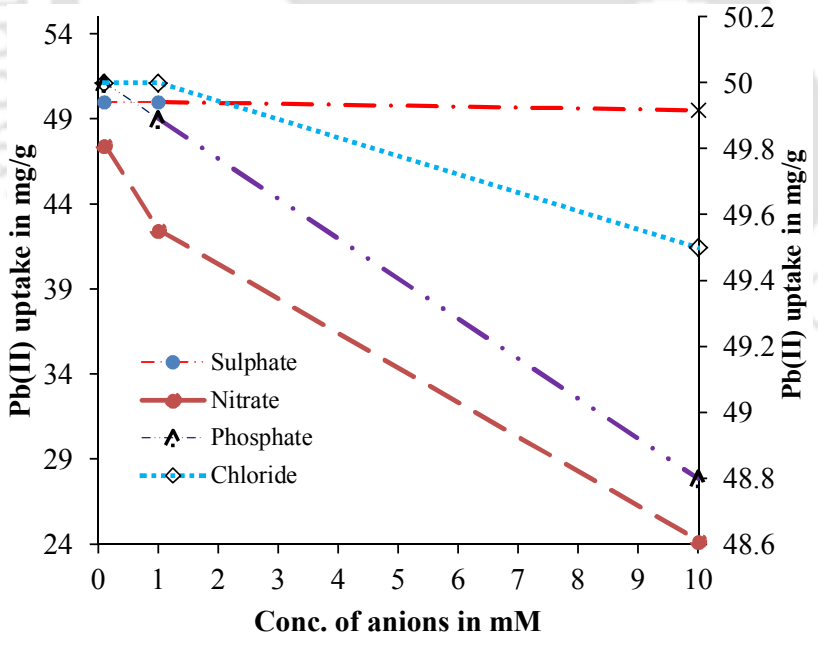


Figure 5.31: Change in Pb(II) uptake in mg/g with different anion conc. (Biomass dosage of 2 g/L, initial metal conc. of 100 mg/L, pH 5, temp. 40°C, 150 rpm and contact time of 2.5 h)

In the present study there was no enhancement in the metal uptake due to SO_4^{2-}, PO_4^{3-} and Cl^- but there was a sharp decrease in uptake at very concentration of nitrate which might be due to the formation of some complexes. Wong et al. (1993) also reported that the presence of anions such as borate, carbonate, chloride and sulphate did not affect the copper uptake by *Pseudomonas putida* II-11.

5.2.3 Effect of Dried Biomass of *Bacillus badius* AK on Actual Water Sample

The water sample was collected from a borewell located at Adabari (26°09'38.5"N, 91°40'58.3"E) Guwahati, India (Figure 5.32). The ground water was reported to have been contaminated there. The initial characterization was carried out in the laboratory and the results are tabulated in Table 5.12. In order to find the practicality of the biomass the actual water sample. The lead concentration was found to be very low in the actual water sample so lead concentration was increased to 5, 10, 15 and 40 mg/L. The pH of each sample was adjusted to 5 and a biomass dosage of 2 g/L was given at 40°C and 150 rpm with a contact time of 2.5 h. The final concentration after biosorption was found out.

Figure 5.32: Location of the sampling area

It was observed that *Bacillus badius* AK was effective in removal of Pb(II) from the water sample. 100% efficiency was achieved for 0.07, 5 and 10 mg/L of Pb(II) samples and 99.66 and 97.08% for 15 and 40 mg/L samples respectively (Figure 5.33). This proved that biomass was effective in removal of Pb(II) even in the presence of other heavy metals,

cations and anions.

Table 5.12: Characteristics of the water sample collected at Adabari, Guwahati.

Sl no.	Parameters	Concentration (mg/L)	Permissible limits(mg/L) BIS 10500:2012
1	Na^+	18.18	-
2	$Ca_2{}^+$	15.89	75
3	K^+	2.89	-
4	Sulphate	6.83	200
5	Nitrates	0.18	45
6	Lead	0.07	0.01
7	Iron	1.36	0.3
8	Cadmium	7.22	0.003
9	Zinc	0.04	5
10	Arsenic	0.0016	0.01

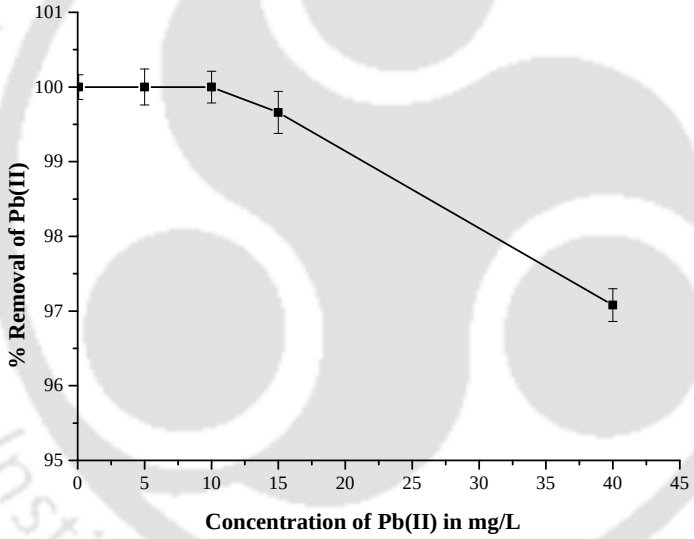
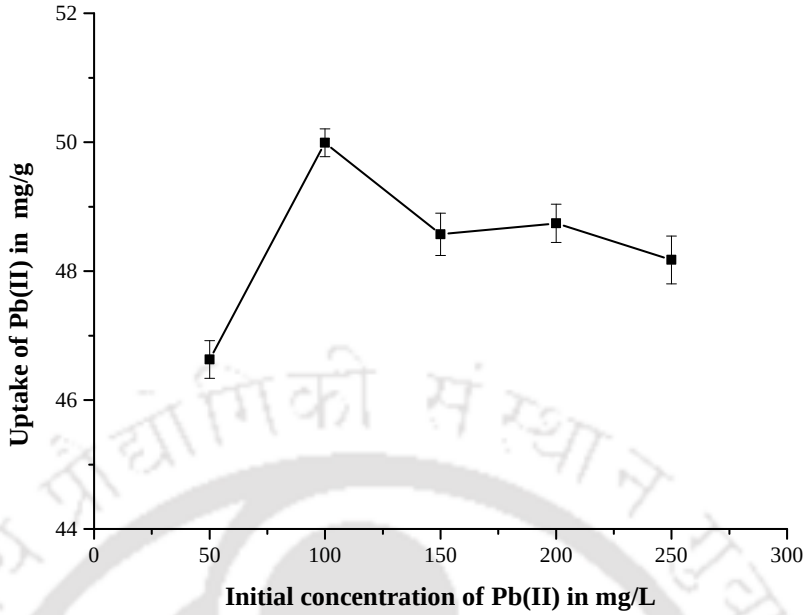


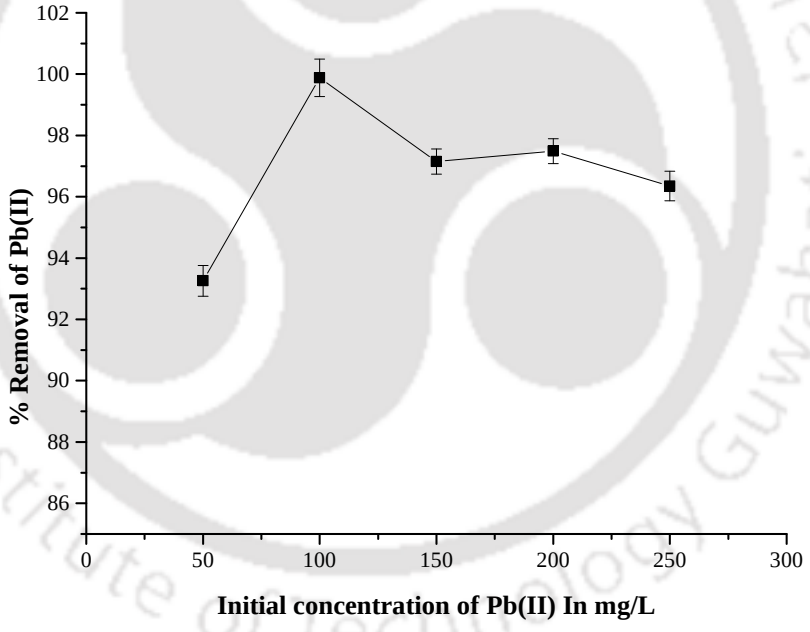
Figure 5.33: Change in percentage removal of Pb(II) from actual water sample (Different conc. of Pb(II) at pH 5, biomass dosage 2 g/L, 150 rpm and contact time of 2.5 h)

• Effect on metal uptake by changing the biomass dosage at different metal concentration

The biomass dosage of 2 g/L was optimized for the removal of Pb(II) at 100 mg/L initial concentration for 2.5 h. In order to find out the authenticity of the result the dosage of biomass was varied with the initial concentration of Pb(II) so that we know the loading rate of the biomass. The tests were carried by adjusting the biomass dosage and initial



(a)



(b)

Figure 5.34: (a) Variation in Pb(II) metal uptake rate (mg/g) at varying initial Pb(II) conc. (mg/L). (b) Change in percentage removal of Pb(II) from actual water sample.

Pb(II) conc. as shown in Table 5.13. The results indicate that the percentage removal and Pb(II) metal uptake by *Bacillus badius* AK was almost similar to results obtained from a biomass dosage of 2 g/L with an initial metal concentration of 100 mg/L (Figure 5.34a and

Figure 5.34b). The metal uptake at 50 mg/L was 46.63 mg/g which was less as compared to 100, 150, 200 and 250 mg/L of initial lead concentration. This might be due to less availability of metal ions as compared to the binding sites. This study proves that a biomass dosage of 2 g/L is optimum for removal of Pb(II) from the aqueous solution.

Table 5.13: Variation in Biomass dosage v/s initial Pb(II) concentration

Biomass dosage g/L	1	2	3	4	5
Initial Pb(II) conc. (mg/L)	50	100	150	200	250

5.2.4 Conclusion

The present study indicated that dried or preteated form of *Bacillus badius* AK can be used as an efficient biosorbent material for the removal of Pb(II) from aqueous solutions. The removal rate was rapid in the first 30 min, and further studies indicated the influence of contact time, temperature, initial biomass dosage and initial metal concentration. The biosorption followed Pseudo second order kinetics. The adsorption isotherm can be described by Langmuir isotherm model indicating possibility of monolayer adsorption. The heat of adsorption was very low indicating possibility of physical adsorption. The maximum adsorption capacity was 138.9 mg/g at an initial concentration of 100 mg/L. Interactions between the metal ions and the functional groups on the surface was confirmed by SEM and EDX spectra. The data from FTIR analysis indicated the presence of several functional groups on the surface of the biomass and their participation in the biosorption process. Unlike the previous study of Pb(II) removal by living cells of *Bacillus badius* AK the dried bacterial biomass was faster in action. The observations from the current study intrigued the authors to use the dried biomass of *Bacillus badius* AK for investigating the removal of Cd(II) in batch biosorption mode in the next study to examine the versatility of this biosorbent.

5.3 PHASE III: Biosorption of Cd(II) by Dried Biomass of *Bacillus badius* AK Strain Isolated from Water Hyacinth Compost

Cadmium (Cd(II)) is present in earth's crust at a concentration of 0.1-0.5 ppm in common association with zinc, lead and copper, it is also present naturally in the ocean waters with an average levels of 5-110 ng/L (Morrow, 2001). Common sources of heavy metal contamination are mining, industrial wastes, batteries, plastics, fertilizers, vehicle emissions, paints etc. (Waalkes, 2000; Oh et al., 2009).

Along with lead and mercury cadmium has a major deleterious impact on the environment and human health (Volesky, 1994). According to The International Agency for Research on Cancer and the US National Toxicology Program cadmium is the human carcinogen, the prolonged exposure to cadmium can result into dysfunctions of kidney, liver and central nervous system (UNEP, 2015). WHO has recognized that the average weekly intake of cadmium present in food lies in the range of 0.7-2.8 μg/kg of body weight (WHO, 2011). Modes of exposure to cadmium include air, water, food, soil, consumer products. Plants and invertebrates can accumulate cadmium, predators feeding on it can introduce cadmium into the food chain, causing bioaccumulation in the ecosystem. According to EPA maximum contaminant level for cadmium exposure is 0.005 mg/L. WHO recommended in its guidelines, 0.003 mg/L of Cd as threshold limit in drinking water (WHO, 2011).

Conventional pysico-chemical techniques adopted to remove heavy metals from wastewaters include ion exchange, precipitation, coagulation, electroplating, membrane process and adsorption. However, these applications have technological and economical constraints. Large amounts of chemical reagents and secondary products are released in these processes, which are difficult to remove. Emerging trend of biosorption process for the heavy metal removal is proving to be an efficient technique of micro-bioremediation. Usage of biomass including microbes (bacteria, cyanobacteria, fungi and yeasts etc.), algae, natural residues (sawdust, barks, crab shell etc.), industrial wastes (food wastes, fermentation residue, activated sludge etc.), agricultural wastes (wheat bran, rice husk, fruits and vegetable waste etc.) is practised in the biosorption process. The phenomenon of biosorption is seen to be a good alternative to the existing methods as it does not leave any chemical sludge, highly selective, cost-effective, easy to operate and more efficient for treatment of wastewaters in higher volumes (Hawari and Mulligan, 2006; Tunali et al., 2006; Volesky, 2007; Oh et al., 2009; Li et al., 2010; Oves et al., 2013; Fomina and Gadd, 2014; Aryal and Liakopoulou-Kyriakides, 2015). Biosorption can be performed either by dead biomass or the living biomass in passive mode as a metabolically-independent physiochemical mechanism on the cell wall and intracellular interaction of metal ions and the functional groups present on the cell (Volesky, 1994; Fomina and Gadd, 2014). Different biomasses originating from

soil, industrial wastes and wastewaters have been exploited in live and dead conditions for the removal of metal, such as *Pseudomonas stutzeri, Bacillus thuringenesis, Bacillus subtilis, Bacillus* sp., *Bacillus* sp. L14, *Streptomyces ciscaucasicus*, *Streptoverticillium cinnamoneum*, *Bacillus licheniformis* (Hossain and Anantharaman, 2006; Tunali et al., 2006; Zhou et al., 2007; Oh et al., 2009; Guo et al., 2010; Li et al., 2010; Oves et al., 2013).

In the current study dried biomass of *Bacillus badius* AK was chosen as biosorbent for removal of cadmium. *Bacillus badius* AK strain was tested for Pb(II) removal in previous study. The efficiency of removal was good in case of dead or dried biomass as compared to the live biomass. Consequently, for this study, dried biomass of *Bacillus badius* AK was used as a biosorbent for Cd(II) removal. Given this context, the objectives of the current work are to investigate and evaluate the various factors effecting the removal of Cd(II) by dead biomass of *Bacillus badius* AK. Biosorption capacity, kinetics, isothermal study, desorption and reusability of the biomass have also been discussed in anticipation of utilization of biomass on large scale process.

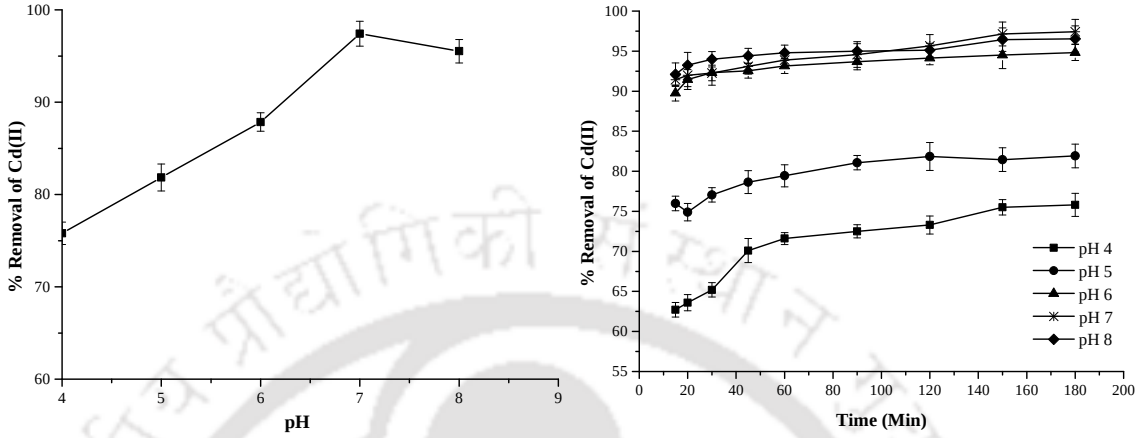
5.3.1 Batch Biosorption Studies

• Effect of solution pH

The pH of metal solution governs the speciation of metal and also the functional groups on the adsorbent. pH value decides the concentration and availability of H+ ions which compete with metal ions for binding on the adsorption sites (Gong et al., 2005; Tunali et al., 2006). Increase in pH enhances the removal of cationic metals as more ligands become available for metal ion binding (Fomina and Gadd, 2014).

In order to study the effect of pH on Cd(II) removal by dead *Bacillus badius* AK biomass, experiments were conducted for pH values ranging from 4 to 8. Figure 5.35a reveals that variation of initial pH leads to increase in biosorption rate of Cd(II) (70-82%) when the pH value varies from 4 to 5. After this pH, increase in Cd(II) removal (87-97%) was observed from pH 6 to 7, thereafter a decrease in Cd(II) biosorption was observed at pH 8 with maximum removal value of 95%, an optimal value of pH 7 was observed. On analyzing the results by ANOVA, a significant variance was observed among all the trials ($p < 0.05$). Other bacterial species have also been reported to perform maximum removal at pH range 6-8 (Esposito et al., 2002; Oh et al., 2009). It was observed that precipitation of Cd(II) started beyond pH 7.6, this being the critical value of precipitation for Cd(II). However, decrease in biosorption above pH 7 is attributed to the lower polarity of cadmium ions at higher pH values, whereas minimum adsorption at lower pH values is due to the ionic competition among the metal ions and the hydrogen ions to attach on the ligands on bacterial cell

surface. Additionally, an observation was made by other authors as distinguishing between sorption and precipitation becomes difficult beyond the critical pH of precipitation (Sheng et al., 2004; Hawari and Mulligan, 2006; Oh et al., 2009).



(a) (b)

Figure 5.35: (a) Effect of pH on Cd(II) removal (b) Effect of contact time on percentage removal of Cd(II) by *Bacillus badius* AK at agitation speed of 150 rpm, initial biomass dosage of 2 g/L and initial metal concentration of 100 mg/L

- **Effect of contact time**

The contact time plays a vital role in interaction of metal ions with the adsorbent sites. Initially all the sites on the adsorbent's surface are vacant and the metal ion concentration is comparatively high. Consequently adsorption rate rises frequently in the initial stage but after some time it decreases and becomes constant thereafter (Azouaou et al., 2010; Çolak et al., 2011). Experiment to determine the equilibrium time of biosorption was performed at the desired time intervals of 0 to 180 min. In the current study, the change in biosorption removal was observed for a time period of 180 min. Observations were made after every 15 min for the first 1h and in the interval of 30 min thereafter. Significant differences were observed between all the triplicates ($p < 0.05$). More than 90% of cadmium removal was achieved in the first 30 min within pH range of 6-7 (Figure 5.35b). Similarly a maximum cadmium removal capacity was achieved within 20 min by bacterial strains *Pseudomonas aeruginosa* CA207Ni, *Burkholderia cepacia* AL96Co and *Corynebacterium kutscheri* FL108Hg, and *Rhodococcus* sp. AL03Ni (Oyetibo et al., 2013). Optimum contact time of 30 min was reported for cadmium removal with the anaerobic granular biomass (Hawari and Mulligan, 2006). Equilibrium time of 12 h was reported for cadmium removal by the *Pseudomonas plecoglossicida* biomass (Guo et al., 2010).

• **Effect of initial biomass dosage**

A series of experiments addressing the effect of biomass dosage on the Cd(II) biosorption efficiency were performed at biomass dosage varying as 0.5, 1, 2, 3 and 4 g/L. Biosorption capacity increases with the increase in biomass concentration as the number of binding sites increases. Figure 5.36 reveals that removal of Cd(II) increases with increase in biomass dosage but becomes constant after 2 g/L of biomass. ANOVA analysis with ($p < 0.05$) depicted significant differences among all the triplicates. It has been mentioned in some studies that biosorption capacity decreases with increasing biomass concentration due to the limitation on mobility of ionic species, the metallic ions remain insufficient in solution as compared to the binding sites (Fourest and Roux, 1992; Tangaromsuk et al., 2002). Higher uptake at lower biomass concentration could be because of the enhanced metal-to-biosorbent ratio, as with excessive increase in biomass concentration the ratio gets disturbed resulting into lowering of biosorption (Puranik and Paknikar, 1999).

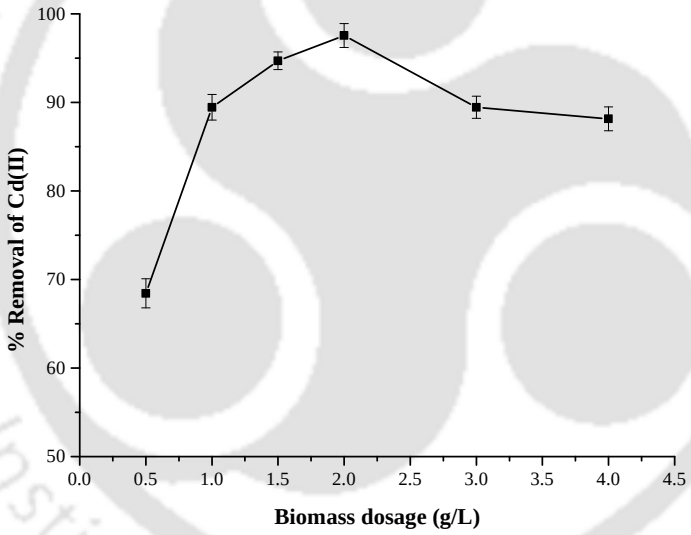
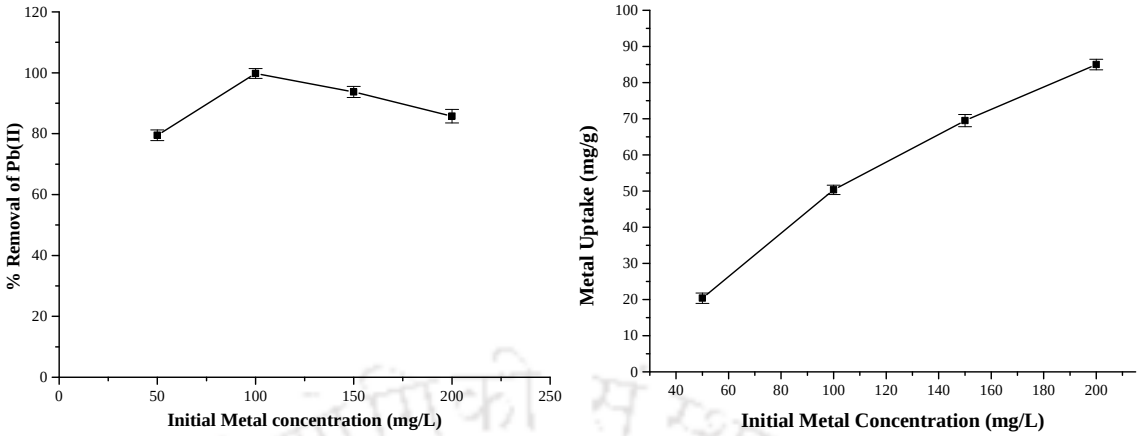


Figure 5.36: Percentage removal of Cd(II) with varying initial biomass dosage

• **Effect of initial metal concentration**

The effect of initial cadmium concentration on biosorption capacity of dried biomass of *Bacillus badius* AK was investigated in the range of 50-200 mg/L of Cd(II) solution. As depicted in Figure 5.37a and 5.37b the percentage removal and uptake was lower at 50 mg/L with thereafter increased at 100 mg/L of Cd(II) concentration. Similarly metal uptake enhancement was observed during biosorption of Cd(II), Pb(II) and Zn (II) by the bacterial strain *Citrobacter* Strain MCMB-181 (Puranik and Paknikar, 1999). Lower uptake



(a) (b)

Figure 5.37: (a) Change in percentage removal of Cd(II) at varying metal conc. (b) Change in Cd(II) uptake (mg/g) at varying Cd(II) conc. at biomass dosage 2 g/L, pH 7, temp. 40°C

at higher metal concentration is attributed to the increase in metal concentration to the surface area ratio. For an adsorbent of fixed concentration the adsorbent sites are limited to the increasing concentration of the metal ions, thus it results in lowering of metal uptake at higher metal concentration (Azouaou et al., 2010). Similar results were obtained by many researchers (Lu et al., 2006; Tunali et al., 2006; Oh et al., 2009). On analyzing the results by ANOVA, a significant variance was observed among all the trials ($p < 0.05$).

- **Effect of temperature and thermodynamics study**

The rate of biosorption is affected by the change of temperature of the metal solution. Increase in temperature leads to the increase in surface activity and kinetic energy of the solute molecules, but much higher temperature leads to the destruction of the binding sites on the adsorbent's surface (Marqués et al., 1991; Aryal and Liakopoulou-Kyriakides, 2015). In the present study the optimum temperature for biosorption by dried *Bacillus badius* AK biomass was tested by varying temperature from 20 to 50°C for pH 7 with 2 g/L of biomass dosage and 100 mg/L of Cd(II) for 180 min. As depicted in Figure 5.38a and 5.38b almost 90% removal and uptake of Cd(II) ions was found within 30 min of the process, increase in removal was achieved at 30 and 40°C both, with maximum of 97.8% in case of 40°C and 96.4% at 30°C. Thus, 40°C was concluded as the optimum temperature for Cd(II) removal by *Bacillus badius* AK biomass. A maximum of 87.8% removal was achieved at 50°C, which started decreasing after 120 min of contact time. Most of the literatures have mentioned 20 to 40°C as optimum range for metal removal by bacterial species (Oves et al., 2013; Oyetibo et al., 2013; Aryal and Liakopoulou-Kyriakides, 2015). Significant differences were observed between all the triplicates ($p < 0.05$). The observed trend of metal removal shows

the kinetic behaviour of biosorption is controlled by the endothermic process, however higher temperature can lead to the distortion of the binding sites, thus adsorption is reduced accordingly (Zhang et al., 2013). The complex path of biosorption process involve the heat and energy, it can be explained by the thermodynamic parameters. The thermodynamic parameters such as the free energy change (ΔG°), enthalpy change (ΔH°) and entropy change (ΔS°) were calculated to estimate the feasibility and spontaneity of the biosorption process (Eq. 3.14 and Eq. 3.15).

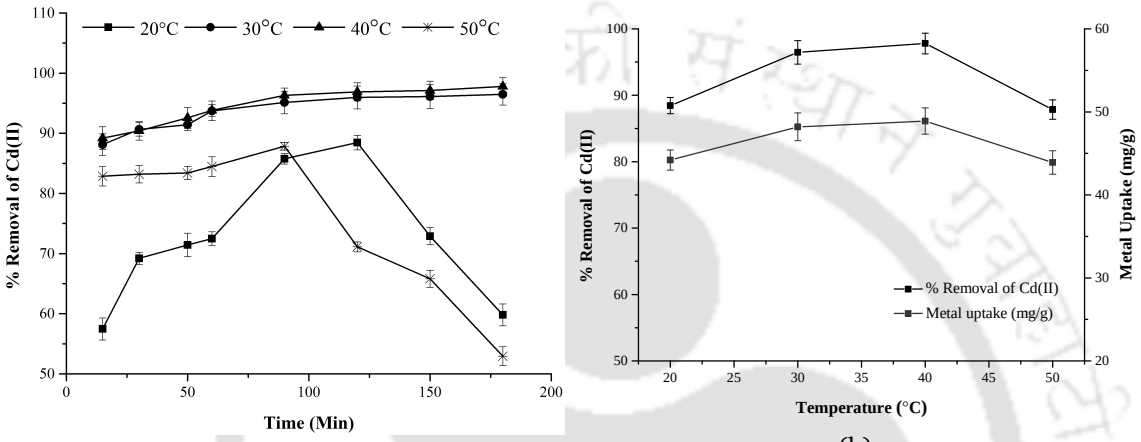


Figure 5.38: (a) Change in percent removal of Cd(II) with time at different temperatures (b) Effect of temperature on removal and uptake of Cd(II) at pH 7, temperature 40°C, biomass dosage 2g/L

Table 5.14 summarizes the thermodynamic parameter of Cd(II) solution with initial concentration of 100 mg/L and biomass dosage of 2 g/L. Positive value of ΔH° concluded the endothermic behaviour of adsorption, negative ΔG° value indicated the spontaneous nature of the adsorption process. Positive value of ΔS° implies the enhanced randomness at the adsorbent-solution interface during the adsorption process. The biosorption heat (ΔH°) for Cd(II) removal was found to be 0.0689 KJ/mol. The enthalpy value of ΔH° gives an indication on the type of adsorption, which can either be physical or chemical. The heat of adsorption value ΔH° ranging between 0 to 20 KJ/mol indicates the consistency of electrostatic interaction between the adsorption sites and the metal ions (physical adsorption), while the heat of chemisorption falls in the range of 80-200 KJ/mol which depicts that the adsorption process involves charge sharing or transfer of metal ion from adsorbent surface to form a coordinate bond (Weng and Wu, 2011; Goswami and Purkait, 2012). Table 5.14 shows that ΔG° decreased from -72433.86 J/mol to -79857.36 J/mol with the increase in temperature from 20°C to 50°C, this suggests that adsorption of Cd(II) by *Bacillus badius* AK was physical adsorption. Similar results were found by Zhang et al. (2013) for removal

of Pb(II) in aqueous solution by *Bacillus gibsonii* S-2 waste biomass , the ΔH° and ΔS° were 23.1 KJ/mol and 87.46 J/mol/K and ΔG° being -2.57 KJ/mol.

Table 5.14: Thermodynamics parameters for Cd(II) biosorption of Cd(II) (100 mg/L)

Biomass dosage (g/L)	**Temperature (°C)**	ΔH° **(J/mol)**	ΔS° **(J/mol.K)**	**-**ΔG° **(J/mol)**
	20			-72433.86
2	30			-74908.36
	40	68.99224	247.4534	-77382.86
	50			-79857.36

• **Kinetic study**

In order to find the kinetics of Cd(II) adsorption onto *Bacillus badius* AK, Lagrange's pseudo first order and second order kinetic models were applied. From the linear form of the first order model (Eq. 3.8)). R^2 value at 100 mg/L was 0.38. First order rate constant (K_1) and theoretical q_e values (q_{em}) were calculated using method of least squares and the obtained parameters are listed in Table 5.15. Similarly, the obtained experimental data were fitted with of pseudo second and first order model as shown in Figure 5.39.

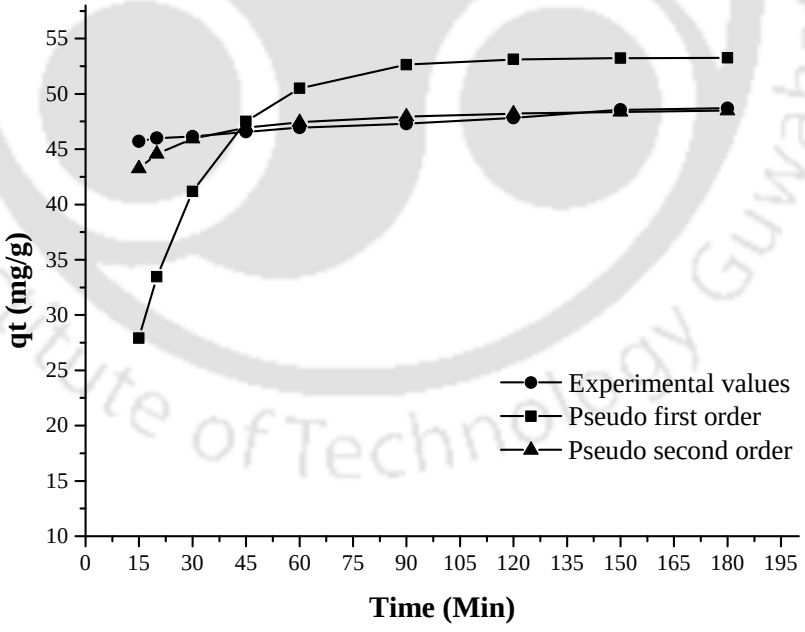


Figure 5.39: Plots of pseudo first order, second order and experimental data for Cd(II) biosorption

The initial metal concentration was kept at 100 mg/L, biomass dosage 2 g/L, temperature 40°C and an agitation of 150 rpm. From kinetics study it was observed that equilibrium was

reached after 2.5 h. From the table it was clear that R^2 value was (0.99) in pseudo second order and moreover the equilibrium adsorption capacity calculated using pseudo second order model was in close agreement with the experimentally obtained values. These results suggest that pseudo second order kinetic model is better than the pseudo first order kinetic model in representation of the kinetics of the biosorption system. The pseudo second fitted better for the biosorption system when *Bacillus gibsonii* S-2 waste biomass was used for removal of Pb(II) from the aqueous solution (Zhang et al., 2013).

Table 5.15: Comparison of pseudo first order and second order kinetics model

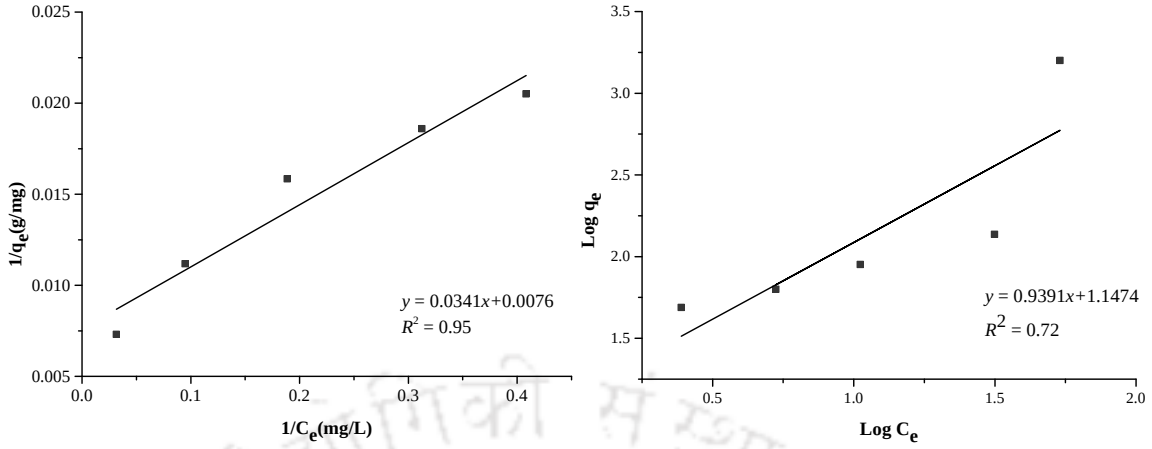
Initial conc. of Cd(II) (mg/L)	q_e**,exp (mg/g)**	**Pseudo first order kinetics**			**Pseudo second order kinetics**		
		k_1 (/min)	q_e,cal (mg/g)	R^2	q_e,cal (mg/g)	k_2 (g/mg.min)	R^2
100	48.72	0.049	53.25	0.38	49.01	0.0102	0.99

- **Isotherm study**

The prediction of adsorption behaviour of *Bacillus badius* AK was performed using Langmuir and freundlich isotherm models. The results are shown in Table 5.16. The initial metal concentration was 100 mg/L. By comparing the correlation coefficients, it can be concluded that Langmuir model fitted better than Freundlich model, the R^2 value is higher for Langmuir model (Figure 5.40a and 5.40b). As Langmuir model provides a good model for the biosorption system it can be characterized as a monolayer single type phenomenon with no interaction between the metal ions (Langmuir, 1918). The maximum adsorption capacity q_{max} was 131.58 mg/g and the constant b (which is the ratio of adsorption rate constant to the desorption rate constant) showed a high value, thereby indicating high affinity of the metal ions for the binding sites on the adsorbent (Volesky and Holan, 1995; Tunali et al., 2006).

Table 5.16: Comparison of Langmuir and Freundlich isotherms

Initial concentration of Cd (mg/L)	Langmuir isotherm			Freundlich isotherm		
	R^2	q_{max} (mg/g)	b(L/mg)	R^2	n	K_F (mg/g)
100	0.945	131.58	0.22	0.719	1.06	3.15



(a) (b)

Figure 5.40: (a) Langmuir isotherm plot of Cd(II) biosorption by *Bacillus badius* AK (b) Freundlich isotherm plot of Cd(II) biosorption by *Bacillus badius* AK.

The value of dimensionless equilibrium parameter R_L which is defined as was observed to be 0.043, it is in the range of 0< R_L<1 which reflects a favourable adsorption process (Hall et al., 1966; Azouaou et al., 2010).

- **Desorption, recovery and reuse**

After the biosorption process, the biosorbent becomes metal-loaded, the metal ions attached on the surface should be recovered for the reuse of biomass. The reuse of biosorbent is important for its practical applications. In the current experiment Cd(II) was desorbed after biosorption with different desorbents such as 0.1M EDTA, HCl, HNO_3, CH_3COOH and H_2SO_4 (Deng et al., 2007). Figure 5.41 shows the percentage of Cd(II) released after the treatment with different desorbents. Attempts were made to desorb Cd(II) with distilled water, however negligible recovery was observed. The initial pH of each desorbent was noted and a total time of 3.5 h was provided initially for desorption. It was observed that 0.1M HCl, H_2SO_4 and HNO_3 gave more than 90% recovery of Cd(II). But desorption with HCl was achieved in less time as compared with other desorbents i.e. within 2.5 h as compared to H_2SO_4 and HNO_3 which was 3.5 h for them. The recovery with 0.1M CH_3COOH and EDTA was 74.8 and 72.3% respectively, whereas negligible recovery was observed with distilled water. The biosorbent is suitable for reuse after recovery by HCl.

The recovery of biosorbent after biosorption was 39% of the initial value and the recovery after desorption was 68.2% (all these values are reported after considering the losses during recovery). The reuse of biosorbent was performed after desorption study with optimum conditions of 100 mg/L of Cd(II), temperature 40°C, biomass dosage of 2 g/L, pH 7 and agitation speed of 150 rpm. Results indicated that percentage removal of Cd(II) was 52%,

this might be due the destruction of sites at the surface of the biosorbent during desorption. Similar decrease in biosorption after desorption with acid (0.1M HCl) was observed during biosorption of Pb(II), Cd(II) and Zn (II) by *Citrobacter* strain MCMB-181, they attributed the decrease to the deleterious effect of acid on the biomass (Puranik and Paknikar, 1999).

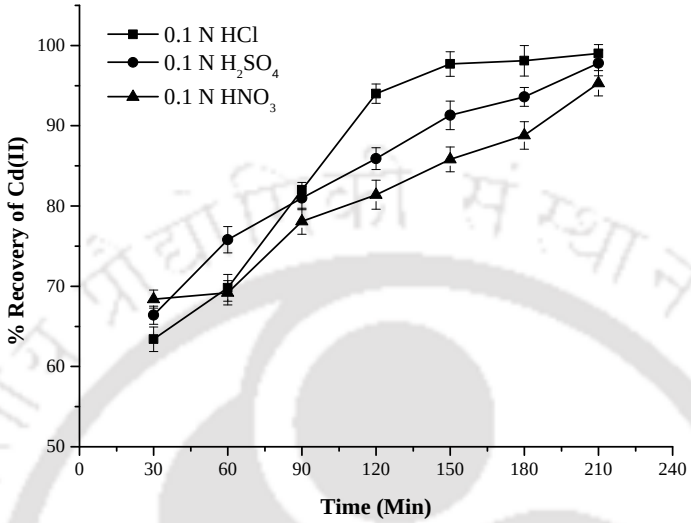


Figure 5.41: Percent recovery of Cd(II) using different desorbents

This study indicated that the bacteria *Bacillus badius* AK which was isolated from rotary drum compost of water hyacinth can be used effectively as a biosorbent for the removal of Cd(II) from aqueous solutions. Within first 30 min maximum removal was observed and further studies indicated the influence of surrounding parameters such as contact time, temperature, initial biomass dosage and initial metal concentration. The biosorption process followed the Pseudo second order kinetics model. The Langmuir isotherm can be described by Langmuir isotherm model depicted the monolayer adsorption nature of the biodsorbent. The maximum adsorption capacity was 131.58 mg/g. The low adsorption heat portrayed that the nature of adsorption was physical adsorption.

5.3.2 Conclusion

The current study indicated that the dried biomass of *Bacillus badius* AK can be used effectively as a biosorbent for the removal of Cd(II) from aqueous solutions in batch system. The optimum conditions of biosorption were determined to be pH 7, contact time of 30 min, initial biomass dosage of 2 g/L at a constant temperature of 40°C, the initial metal concentration of 100 mg/L and agitated at 150 rpm. Within first 30 min, maximum removal was observed and further studies indicated the influence of surrounding parameters such as contact time, temperature, initial biomass dosage and initial metal concentration. The biosorption process followed the pseudo second order kinetics model. The Langmuir isotherm can be described by Langmuir isotherm model depicted the monolayer adsorption nature of the biosorbent. The maximum biosorption capacity was 131.6 mg/g. The results indicated that the bacterium *Bacillus badius* AK was effective in the removal of Cd(II) which could be advantageous in the large-scale treatment of the contaminated wastewater. However, the biosorption capacity of dried *Bacillus badius* AK for Cd(II) was lower than that for Pb(II) which was 138.9 mg/g. Considering the results the dried *Bacillus badius* AK biomass was investigated for biosorption of Pb(II) in continuous column mode operation in the next study.

5.4 PHASE IV: Biosorption Studies of Pb(II) Dead Biomass in Column Mode Operation

Batch data has several limitations to predict the performances in a practical situation such as industrial wastewater treatment plant, where continuous reactors replace batch operation. In the present work, dried biomass of *Bacillus badius* AK was used for removal of Pb(II) in continuous mode. Influent Pb(II) concentration, influent pH, and volumetric flow rate were kept constant at 100 mg/L, 5 and 1 mL/min respectively. Columns of 1 cm diameter were used for different biomass bed depths. In continuous mode, bed height was a variable parameter.

To study the effect of bed depth, experiment were conducted with three columns filled with 0.88, 1.32, 1.77 g of dried *Bacillus badius* AK to yield respective bed depth of 1, 1.5 and 2 cm respectively. Influent Pb(II) concentration of 100 mg/L with pH 5 was fed at constant flow rate of 1 mL/min. Details of breakthrough time, exhaustion time, breakthrough volume, exhaustion volume along with the amount of total Pb(II) removed and uptake are presented in Figure 5.42 and Table 5.17 For the bed depths of 1, 1.5 and 2 cm the throughput volume at the breakthrough point of 50% of initial Pb(II) concentration i.e. 100 mg/L during adsorption was 0.09, 0.12 and 0.19 L respectively and their corresponding exhaust volumes were 0.27, 0.37 and 0.51 L respectively. At lower bed depth, the governing mass transfer phenomenon was the axial dispersion of the lead ions which reduced the diffusion of Pb(II) ions (Taty-Costodes et al., 2005). Additionally, with the increase in bed depth mass transfer zone also got broadened as well as more binding sites were available for adsorption (Kumar and Chakraborty, 2009). Thus Pb(II) mass removed (M_r) were evaluated using Eq. 3.21 and Eq. 3.22 for column depth of 1, 1.5 and 2 cm and were observed as 8.13, 13.3 and 20.09 mg respectively.

Table 5.17 shows that Pb(II) uptake by *Bacillus badius* AK for bed depth of 1, 1.5 and 2 cm was 9.24, 10.10 and 11.35 mg/g respectively. With the increase in bed depth, the contact time between the adsorbate and the adsorbent increased as a result more number of adsorption sites are available resulting increase in q_e value (Kumar and Chakraborty, 2009; Long et al., 2014). Also with the increase in bed height, the surface area is increased accordingly, providing more binding sites for adsorption (Zulfadhly et al., 2001). In the present study, the metal removal efficiency increased with the increase in bed height but the biosorption capacity or the breakthrough adsorption column capacity remained unaffected by the bed height increment. Similar results were reported by (Singh et al., 2012; Mondal, 2009) for the metal removal by *Spirogyra* packed fixed bed column and activated tea waste respectively. However, an uptake (q_e) value obtained from batch study process showed 138.8 mg/g, much higher than uptake value observed in column work. The break-

Table 5.17: Effect of column bed depth on removal of Pb(II) by *Bacillus badius* AK

Bed depth (cm)	Biomass amount (g)	t_B (min) 0.10%	t_B (min) 50%	t_E (min)	V_B 0.1% (L)	V_B 50% (L)	Influent Adsorbate Load (I) (mg)	Amount metal removed [M_r] (mg)	Metal uptake [q_e] (mg/g)	Breakthrough capacity or adsorption column capacity at 50 % breakthrough (mg/g)
1	0.88	94.8 (1.58 h)	265	0.02	0.09	0.27	26.5	8.13	9.24	10.77
1.5	1.32	121.78 (2.03 h)	370	0.024	0.116	0.37	37	13.33	10.1	9.23
2	1.77	195.4 (3.26 h)	540	0.042	0.196	0.51	51	20.09	11.35	11.05

t_B is breakthrough time, t_E is exhaustion time, V_B is breakthrough volume, V_E is exhaustion volume

through and exhaustion time at 2 cm were 3.26 h and 9 h respectively. Additionally, the breakthrough capacity of the biosorbent is higher in the case of column process as compared to the batch process. This is because of the decreasing concentration gradient in the batch process than the continuously increasing large concentration in the column mode operation (Çolak et al., 2011). Beyond a bed height of 2 cm, no appreciable changes were observed in effluent concentration with further increase of bed height, hence 2 cm was chosen as the optimized bed height for the Pb(II) removal study in column mode (Figure 5.42).

In continuous mode, the residence time of metal ions in contact with the adsorbent was brief, as compared to the batch system. Whereas the batch system ensured the longer duration of exposure time to the metal ions in the solution. On the contrary, the maximum

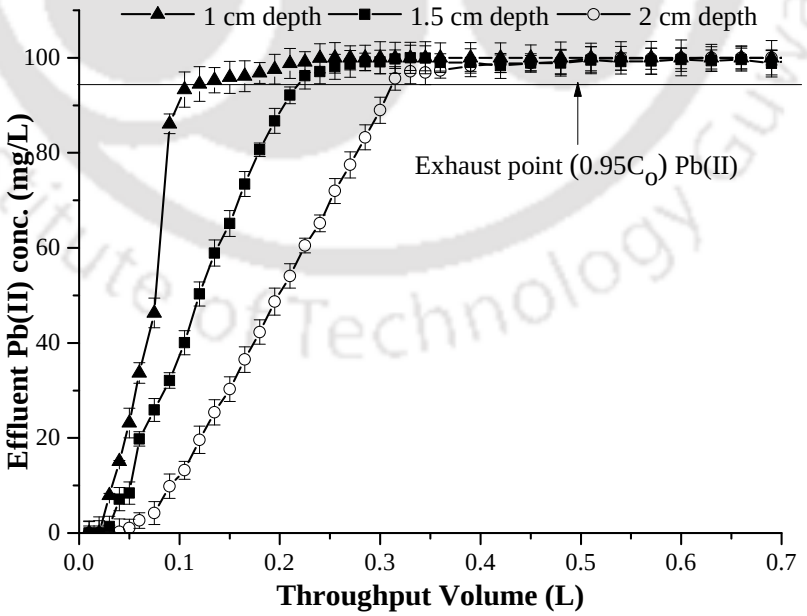


Figure 5.42: Breaktrough curves of Pb(II) during adsorption by dried *Bacillus badius* AK at different column bed depths

biosorption was reported within a span of 30 min. at high flow rate the metal ions get less time for attachment with the binding sites, however, the adsorbent gets exhausted rapidly. While a low flow rate continuous system is impracticable for maintenance, also the uptake capacity in the batch system is above the column mode. Therefore, the approach of a batch system for metal removal by biosorbent *Bacillus badius* AK is better than the column mode.

5.4.1 Bed Depth Service Time (BDST) Model

Service time (ts) and bed depth results are plotted in Figure 5.43 according to equation (3.16) for 0.1%, 10%, 50% and 99% breakthrough at column bed depth of 1, 1.5 and 2 cm. At other breakthrough values of slope, intercepts and correlation coefficients were calculated in a similar way plotting service time and bed depth and results are shown in Table 5.18 proving the validity of BDST model for the fixed bed of *Bacillus badius* AK.

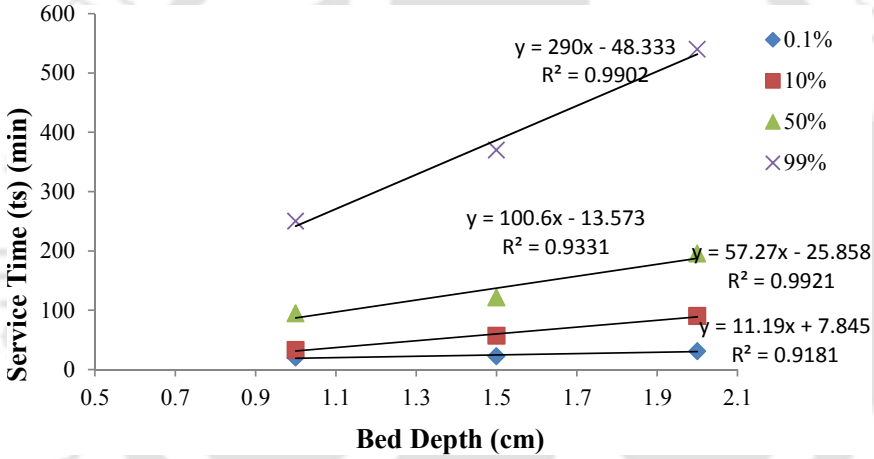


Figure 5.43: BDST plot for Pb(II) biosorption at various breakthrough

Table 5.18 and Figure 5.43 suggest that correlation coefficient values are all above 0.90 suggesting the data fixing on BDST model. There was also a consistent rise in slopes from 11.2 to 290.0 from the breakthrough of 0.1 to 99% and subsequent increase in corresponding dynamic adsorption capacity N_o from 884.01 to 22910 mg/L. At lower breakthrough

Table 5.18: Coefficients of BDST equation at different breakthrough

Breakthrough (%)	Slopes	Intercepts	N_o (mg/L)	K (L/mg h)	Coefficient correlation (R^2)
0.1	11.2	7.8	884	9×10^{-3}	0.92
10	57.3	-25.9	4524.3	8×10^{-4}	0.99
50	100.6	-13.6	7947.4	0	0.93
99	290	-48.3	22910	2×10^{-6}	0.99

value, the adsorbent remains unsaturated as some of the adsorption sites remain unoccupied. In a continuous flow system, the metal adsorption increased with the increasing volume of metal solution. This is attributed to the fact that a larger volume of metal solution passing through the adsorbent column more is the amount of metal ion interacted. Similar results of an increase in slope with an increase in breakthrough values were reported in the literature (Zulfadhly et al., 2001; Mondal, 2009). Whereas, Long et al. (2014) reported a decrease in biosorption capacity (N_o) with the increase in bed height during adsorption of Pb(II) by packed bed column of Agaricus bisporus. It has been found that adsorption rate constant (K_{ads}) decreased from 9×10^{-3} to 2×10^{-6}. This is related to the fact that with the increase in breakthrough value the adsorbent becomes exhausted as a result the rate of adsorption also decreases. Similarly, Long et al. (2014) also reported a decrease in adsorption rate constant with the increase in bed height. Kumar and Chakraborty (2009) observed the lowering of adsorption rate constant from breakthrough values 1-90% during removal of Cr(VI) by jute fiber. All these experimental and literature findings suggest that BDST model is advantageous as the experimental test can be scaled up without any further intervention regarding experimental data and analysis.

5.5 Conclusion

From the study it can be concluded that uptake (q_e) value obtained from batch study process showed 138.8 mg/g, much higher than uptake value observed in column work. The breakthrough capacity of the biosorbent is higher in the case of column process as compared to the batch process. The breakthrough and exhaustion time at 2 cm were 3.26 h and 9 h respectively. A bed height of 2 cm was chosen as the optimized bed height for the Pb(II) removal study in column mode. Beyond 2 cm of bed height no appreciable changes were observed in effluent concentration. The BDST model depicted the consistent rise in slopes from 11.2 to 290.0 from the breakthrough of 0.1 to 99% and subsequent increase in corresponding dynamic adsorption capacity N_o from 884.01 to 22910 mg/L. At lower breakthrough value, the adsorbent remains unsaturated as some of the adsorption sites remain unoccupied. At a high flow rate the adsorbent got exhausted rapidly, while a low flow rate continuous system is impracticable for situation such as industrial wastewater treatment plant, where continuous reactors replace batch operation. Therefore, the approach of a batch system for metal removal by biosorbent *Bacillus badius* AK is better than the column mode operation.

"If we intend to provide a better life, and a better world, for future generations, we can't ignore the quality of the environment we leave them."

John Kasich

6

Conclusions and Recommendations

This chapter deals with the conclusion achieved from all the phases conducted with the rotary drum composting of water hyacinth, isolation and identification of microbes from water hyacinth compost, biosorption studies of Pb(II) and Cd(II) with the isolated bacterial strain in batch and column system

6.1 Conclusions

Rotary drum composting of water hyacinth was successful with combination of cow dung and sawdust. Highest temperature and degradation was found in trial 3 (6:3:1). Bacterial count was highest among all microbes varying from mesophilic to spore forming form according to temperature. Their count remained substantial at the end of composting period. Actinomycetes and streptomycetes count remained higher due to presence of lignocelluloses in water hyacinth. Fungal count reduced as degradation proceeded but it remained till the end. Lower CO_2 evolution rate and OUR in the end signified stability of compost. Volatile solids (%) at end of composting period indicated the metabolic action of microbes on complex lignocellulosic material forming monomeric compounds but not completely into humic substances. Hence, the compost required more maturation and could not be applied to field directly. Further study was carried out on trial 3 (6:3:1) as it was found to be the best amongst all other trials. Also mesophilic bacteria were in abundance throughout the composting period. Therefore, they were taken as target microbes for isolation and metal interaction study. This made it possible to understand the microbial dynamics of water hyacinth composting more profoundly. 16S metagenome sequencing of best trial (6:3:1) of water hyacinth compost was observed with 39.08% of *Bacteroidetes*, 24.21% of *Flavobacteria* followed by 24.21% of *Flavobacteriales*. *Flavobacterium* genus was found to be most abundant in the composting process. The phylum *Bacteroidetes* plays a crucial role

during the initial and final stages of composting in maturation of compost by degrading complex polymers. The *Flavobacteria* and *Sphingobacteria* belong to *Bacteroidetes* species, they contribute for the degradation of starch, cellulose, proteins, chitin, and the phenolic and chlorinated compounds. All the bacterial communities belonging to *Bacteroidetes* are known for degrading lingo-cellulosic compost material, being present during curing of compost as well as in noncured compost.

Glucose minimal medium was found to be the most efficient media for culturing bacterial isolates as compared to the other three media. Twelve new robust bacterial strains belonging to the *Bacillus* and *Enterobacter* family were isolated as being the most consistent throughout the 20 days of the composting period. Both the culture dependent and culture independent approach gave a spectacle of new bacterial strains. The bacterial strains not only survived the rigorous variations of composting process, but also the presence of the toxic heavy metals of the compost. The reduction in water soluble, leachability, and DTPA values of heavy metals in compost indicated the efficient degradation of the organic matter in the presence of microbial community during the composting process. The bacterial species were investigated further for their capability of heavy metal removal for the purpose of micro-bioremediation.

A bacterial strain *Bacillus badius* AK, isolated from rotary drum compost of water hyacinth was investigated for biosorption of Pb(II) and Cd(II) in live (non-pretreated) state and dried (pretreated) state. Batch biosorption study of live (non-pretreated) biomass of *Bacillus badius* AK demonstrated the optimum conditions of pH at 4, temperature at 30°C, 150 rpm of rotational speed at biomass concentration of 20 mL with 1.7×10^{16} CFU/mL value, at 100-150 mg/L concentration of Pb(II). The biosorption followed pseudo second order kinetics and isotherm fitted well to the Langmuir model. The specific growth rate and maximum specific growth rate of bacterial cells under the influence of Pb(II) were determined as 0.05 h^{-1} and 2.54 h^{-1} respectively, with a biomass yield coefficient of 11.81.

The batch biosorption study with dried (pretreated) biomass of *Bacillus badius* AK was rapid in the first 30 min, and further studies indicated the optimum batch conditions to be pH at 5, equilibrium contact time of 2.5 h at constant temperature of 40 ÂřC, initial metal concentration of 100 mg/L and rotational speed of 150 rpm. The maximum adsorption capacity was 138.88 mg/g. The loading rate test proved that the optimum dosage for the biomass is 2 g/L.The biosorption followed pseudo second order kinetics, langmuir isotherm fitted better than the fruendlich isotherm indicating possibility of monolayer adsorption. The heat of adsorption was very low indicating possibility of physical adsorption. The data from FTIR and EDX analysis indicated the presence of several functional groups on the surface of the biomass and their participation in the biosorption process. A 100% desorption of Pb(II) was achieved using 0.1M HCl within 2.5 h but negligible amount of Pb(II) was

recovered using water which indicated that the biosorbent was fit for treating drinking water. Pb(II) removal efficiency was 60% after desorption. Storage study also proved that the biosorbent can be stored for at least 3 months with very less changes in the metal uptake and removal efficiency. There was very little variation in removal efficiency using sulphates, phosphates and chloride, uptake decreased only at very high concentration of nitrates.

The biomass of *Bacillus badius* AK showed promising results when it was tested with actual water sample with removal efficiency reaching as high as 97% with initial lead concentration of 40 mg/L. Biosorption of Cd(II) by dried biomass of *Bacillus badius* AK was investigated for its potential application in biosorption of heavy metal other than Pb(II) in batch system. The optimum conditions of biosorption were determined to as pH adjusted to 7, contact time of 30 min, initial biomass dosage of 2 g/L at a constant temperature of 40°C, the initial metal concentration of 100 mg/L and agitation at 150 rpm. The maximum biosorption capacities of Cd(II) on *Bacillus badius* AK was found to be 131.58 mg/g. The pseudo-second order model fitted better than the first order model. Langmuir isotherm fitting depicted the monolayer adsorption behavior. The low adsorption heat portrayed that the nature of adsorption was physical adsorption. The recovery of biosorbent after biosorption was 39% of the initial value and the recovery after desorption was 68.2%. Reuse of biosorbent after desorption study showed 52% of Cd (II) removal this was due to the destruction of sites at the surface of the biosorbent during desorption. The biosorption capacity of *Bacillus badius* AK for Pb(II) removal was more than that for Cd(II).

The continuous column mode operation of dried biomass of *Bacillus badius* AK in biosorption of Pb(II) was observed with higher breakthrough capacity as compared to the batch process. But at a high flow rate the adsorbent got exhausted rapidly, while a low flow rate continuous system is impracticable for situation such as industrial wastewater treatment plant. This showed that replacement of biosorbent in the column was required to be frequent which is not feasible for a upscale industrial application. Thus, the approach of a batch system for metal removal by biosorbent *Bacillus badius* AK is better than the column mode operation.

Therefore, it can be concluded that rotary drum compost of water hyacinth being a process of microbial degradation of waste organic material serves as an efficient source of robust and vigorous microbes. The isolated microbes justified their potential of surviving in the unfavourable metal loaded conditions of water hyacinth compost by being utilised in biosorption technology for micro-bioremediation. *Bacillus* and *Enterobacter* family were prominent among the isolated bacterial species. *Bacillus badius* AK was found to be most efficient in surviving with its higher growth rate. Biosorption of heavy metals such as Pb(II) and Cd(II) was accomplished in the living (non-pretreated) as well as dried (pretreated) state of bacterial biomass. It was observed that the dried (pretreated) bacterial biomass

performed well in Pb(II) and Cd(II) batch biosorption. The column mode operation gave a spectacle of the application of biosorbent in the industrial process. The efficiency of biosorption was faster and higher as compared to the batch system but due to its higher exhaustion rate it becomes non-feasible and impracticable to use this biosorbent for upscale process. The other technologies for pretreatment of biomass can make the modification of biomass in favour of its application at the industrial level.

6.2 Future Recommendations

- Efficiency of removal for other heavy metals.
- Biomass immobilization of *Bacillus badius* AK with different techniques.
- Application of immobilized biomass in column mode operation.
- Studies on availability and speciation of heavy metals in wastewater due to bacterial biomass.

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Publications Related to Thesis

Journals Published or Accepted

1. Vishan, I., Kanekar, H., Kalamdhad, A.S., 2014. Microbial population, stability and maturity analysis of rotary drum composting of water hyacinth. *Biologia* 69(10) 1303-1313.
2. Vishan, I., Kalamdhad, A.S., 2016. Heavy metal removal through bacterial biomass isolated from various contaminated sites. *International Journal of Environmental Sciences* 7(1), 1-18.
3. Vishan, I., Laha, A., Kalamdhad, A.S., 2017. Biosorption of Pb (II) by *Bacillus badius* AK strain originating from rotary drum compost of water hyacinth. *Water Science and Technology* 7(5) 1071-1083.
4. Vishan I., Kalamdhad A.S., 2017 Biosorption of lead using *Bacillus badius* AK strain isolated from compost of green waste (water hyacinth). *Environmental Technology* 38, 13-14.

16s rRNA Sequences submitted in NCBI

Vishan, I., Sivaprakasam, S., Kalamdhad, A.S., 2015. Submission of 16S rRNA sequences of twelve isolated strains in NCBI https://www.ncbi.nlm.nih.gov/genbank).

1. Accession no: KP216715. 16s rRNA of isolates of *Bacillus badius* AK
2. Accession no: KR780040 16s rRNA of isolates of *Enterobacter* sp. strain AK 1
3. Accession no: KR780041 16s rRNA of isolates of *Enterobacter* sp. strain AK 2
4. Accession no: KR780042 16s rRNA of isolates of *Enterobacter* sp. strain AK 3
5. Accession no: KR780043 16s rRNA of isolates of *Enterobacter* sp. strain AK 5
6. Accession no: KR780044 16s rRNA of isolates of *Bacillus* sp. strain AK 10
7. Accession no: KR780045 16s rRNA of isolates of *Bacillus thuringinesis* AK 7
8. Accession no: KR780046 16s rRNA of isolates of *Bacillus cereus* strain AK 9

9. Accession no: KR780047 16s rRNA of isolates of *Bacillus cereus* strain AK 6
10. Accession no: KR780048 16s rRNA of isolates of *Bacillus subtilis* AK4
11. Accession no: KR780049 16s rRNA of isolates of *Bacillus cereus* strain AK 11
12. Accession no: KR780050 16s rRNA of isolates of *Bacillus* sp. strain AK 8

Journals (Submitted revision/Under review)

1. Vishan I., Kalamdhad A.S., Isolation of bacterial isolates during Rotary drum composting of water hyacinth. *International Journal of Recycling of Organic Waste in Agriculture*, (Revision submitted).
2. Vishan I., Kalamdhad A.S., Biosorption of Cd(II) by *Bacillus badius* AK strain originating from rotary drum compost of water hyacinth, *Journal of Environmental Chemical Engineering* (Under review).
3. Vishan I., Kalamdhad A.S., Biosorption of Pb(II) in column mode operation by biosorbent *Bacillus badius* AK. *Water Science and Technology*, (Submitted).

International Conferences and Proceedings

1. Vishan, I., Kanekar, H., Kalamdhad, A.S., 2013. Microbial community dynamics during composting of water hyacinth. *Proc. International Conference on Advances in Biotechnology and Bioinformatics*(ICABB 2013), 25-27 Nov., Dr. D. Y. Patil Vidhyapeeth, Pune, India.
2. Vishan, I., Senthilkumar, S., Kalamdhad, A.S., 2014. Isolation and biosorption of metal during rotary drum composting of water hyacinth. *Proc. International Conference on Environment and Energy*, 15-17 Dec., Jawaharlal Nehru Technological University Hyderabad, India.
3. Vishan, I., Laha, A., Senthilkumar, S., Kalamdhad, A.S., 2015. Biosorption of Lead (Pb) by Bacillus badius AK strain (KP 216715) during rotary drum composting of Water Hyacinth. *Proc. International Conference on Solid Waste* (ICSWHK-2015), 19-23 May, 2015, Hong Kong Baptist University, Hong Kong.
4. Vishan, I., Senthilkumar S., Kalamdhad, A.S., 2015. Interaction of *Bacillus badius* AK strain with Lead (Pb) isolated during rotary drum composting of water hyacinth.

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5. Vishan, I., Kalamdhad, A.S., 2016. Biosorption of Pb (II) by *Bacillus Badius* AK strain originating from rotary drum compost of water hyacinth. *Proc. International Conference on Waste Management* (Recycle 2016), 1-2 April, Indian Institute of Technology Guwahati, Guwahati, India.

National Conferences and Proceedings

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1. Vishan, I., Kanekar, H., Kalamdhad, A.S., 2014. Microbial succesion, stability and maturity analysis of rotary drum composting of water hyacinth. *Proc. National Conference on Sustainable Development of Environmental System* (NCOSDOES-2014), 20-21 June, Centre for the Environment, Indian institute of Technology Guwahati, Guwahati, India.
2. Vishan, I., Kalamdhad, A.S., 2015. Biosorption of lead by *Bacillus badius* AK strain isolated from water hyacinth compost. *Proc. Management and Procurement of Integrated Waste Management System*, 6-7th Feb., Indian Institute of Technology Guwahati, India. (Best oral presentation award)
3. Vishan, I., Kalamdhad, A.S., 2015. Interaction of Bacillus badius AK strain with Lead (Pb) isolated during rotary drum composting of water hyacinth. *Proc. National Conference on Challenges in Environmental Research* (NCOCER 2015), 4-6 June, Indian Institute of Technology Guwahati, Guwahati, India

Awards

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1. Best Paper Award for presenting the paper entitled "Biosorption of Pb(II) by *Bacillus Badius* AK strain originating from rotary drum compost of water hyacinth" at International Conference on Waste Management (Recycle 2016), 1-2 April, 2016, Indian Institute of Technology Guwahati, India.
2. Best Oral Presentation Award at Management and Procurement of Integrated Waste Management System, 6-7th Feb., 2015, Indian Institute of Technology Guwahati, India.
3. Second Prize for presenting the paper entitled "Microbial and biosorption studies during rotary composting of water hyacinth" on Research Scholar Day, 19 Jan, 2015, Indian Institute of Technology Guwahati, India.